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* * * * * * * * * *   Welcome to STN International   * * * * * * * * * *

 NEWS  1           Web Page URLs for STN Seminar Schedule - N. America
 NEWS  2           "Ask CAS" for self-help around the clock
 NEWS  3  JAN 27   Source of Registration (SR) information in REGISTRY updated
                   and searchable
 NEWS  4  JAN 27   A new search aid, the Company Name Thesaurus, available in
                   CA/CAplus
 NEWS  5  FEB 05   German (DE) application and patent publication number format
                   changes
 NEWS  6  MAR 03   MEDLINE and LMEDLINE reloaded
 NEWS  7  MAR 03   MEDLINE file segment of TOXCENTER reloaded
 NEWS  8  MAR 03   FRANCEPAT now available on STN
 NEWS  9  MAR 29   Pharmaceutical Substances (PS) now available on STN
 NEWS 10  MAR 29   WPIFV now available on STN
 NEWS 11  MAR 29   No connect hour charges in WPIFV until May 1, 2004
 NEWS 12  MAR 29   New monthly current-awareness alert (SDI) frequency in RAPRA
 NEWS 13  APR 26   PROMT: New display field available
 NEWS 14  APR 26   IFIPAT/IFIUDB/IFICDB: New super search and display field
                   available
 NEWS 15  APR 26   LITALERT now available on STN
 NEWS 16  APR 27   NLDB: New search and display fields available

 NEWS EXPRESS  MARCH 31 CURRENT WINDOWS VERSION IS V7.00A, CURRENT
               MACINTOSH VERSION IS V6.0c(ENG) AND V6.0Jc(JP),
               AND CURRENT DISCOVER FILE IS DATED 26 APRIL 2004
 NEWS HOURS    STN Operating Hours Plus Help Desk Availability
 NEWS INTER    General Internet Information
 NEWS LOGIN    Welcome Banner and News Items
 NEWS PHONE    Direct Dial and Telecommunication Network Access to STN
 NEWS WWW      CAS World Wide Web Site (general information)

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  * * * * * * * * * * * * * * STN  Columbus  * * * * * * * * * * * * * * *

FILE 'HOME' ENTERED AT 15:58:13 ON 30 APR 2004

=> file uspatful
COST IN U.S. DOLLARS                                    SINCE FILE      TOTAL
                                                           ENTRY    SESSION
FULL ESTIMATED COST                                         0.42       0.42

FILE 'USPATFULL' ENTERED AT 15:59:07 ON 30 APR 2004
CA INDEXING COPYRIGHT (C) 2004 AMERICAN CHEMICAL SOCIETY (ACS)

FILE COVERS 1971 TO PATENT PUBLICATION DATE: 29 Apr 2004 (20040429/PD)
FILE LAST UPDATED: 29 Apr 2004 (20040429/ED)
HIGHEST GRANTED PATENT NUMBER: US6728968
HIGHEST APPLICATION PUBLICATION NUMBER: US2004083524
CA INDEXING IS CURRENT THROUGH 29 Apr 2004 (20040429/UPCA)
ISSUE CLASS FIELDS (/INCL) CURRENT THROUGH: 29 Apr 2004 (20040429/PD)
REVISED CLASS FIELDS (/NCL) LAST RELOADED: Feb 2004
USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Feb 2004

>>>  USPAT2 is now available.  USPATFULL contains full text of the      <<<
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>>>  original, i.e., the earliest published granted patents or          <<<
>>>  applications.  USPAT2 contains full text of the latest US          <<<
>>>  publications, starting in 2001, for the inventions covered in      <<<
>>>  USPATFULL.  A USPATFULL record contains not only the original      <<<
>>>  published document but also a list of any subsequent               <<<
>>>  publications.  The publication number, patent kind code, and       <<<
>>>  publication date for all the US publications for an invention      <<<
>>>  are displayed in the PI (Patent Information) field of USPATFULL    <<<
>>>  records and may be searched in standard search fields, e.g., /PN,  <<<
>>>  /PK, etc.                                                          <<<

>>>  USPATFULL and USPAT2 can be accessed and searched together         <<<
>>>  through the new cluster USPATALL.  Type FILE USPATALL to           <<<
>>>  enter this cluster.                                                <<<
>>>                                                                     <<<
>>>  Use USPATALL when searching terms such as patent assignees,        <<<
>>>  classifications, or claims, that may potentially change from       <<<
>>>  the earliest to the latest publication.                            <<<

This file contains CAS Registry Numbers for easy and accurate
substance identification.

=> e chang gwong jen/in
E1            3     CHANG GWO JER/IN
E2            2     CHANG GWO YANG/IN
E3            0 --> CHANG GWONG JEN/IN
E4            1     CHANG GWONG JEN J/IN
E5            2     CHANG GYU HWAN/IN
E6            2     CHANG H J/IN
E7            2     CHANG HA Y/IN
E8            1     CHANG HAE C/IN
E9            2     CHANG HAE CHOON/IN
E10           1     CHANG HAE S/IN
E11           6     CHANG HAE SUNG/IN
E12           4     CHANG HAEHIE/IN

=> s e4
L1            1 "CHANG GWONG JEN J"/IN

=> d l1,ti

L1   ANSWER 1 OF 1  USPATFULL on STN
TI     Nucleic acid vaccines for prevention of flavivirus infection


=> d l1,cbib,ab,clm

L1   ANSWER 1 OF 1  USPATFULL on STN
2003:30900 Nucleic acid vaccines for prevention of flavivirus infection.
    Chang, Gwong-Jen J., Fort Collins, CO, UNITED STATES
    US 2003022849 A1 20030130
    APPLICATION: US 2001-826115  A1  20010404 (9)
    PRIORITY: US 1998-87908P  19980604 (60)
    DOCUMENT TYPE: Utility; APPLICATION.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB     The present invention encompasses isolated nucleic acids containing
       transcriptional units which encode a signal sequence of one flavivirus
       and an immunogenic flavivirus antigen of a second flavivirus. The
       invention further encompasses a nucleic acid and protein vaccine and the
       use of the vaccine to immunize a subject against flavivirus infection.
       The invention also provides antigens encoded by nucleic acids of the
       invention, antibodies elicited in response to the antigens and use of
       the antigens and/or antibodies in detecting flavivirus or diagnosing
       flavivirus infection.
CLM    What is claimed is:
       1. An isolated nucleic acid comprising a transcriptional unit encoding a
```

signal sequence of a structural protein of a first flavivirus and an immunogenic flavivirus antigen of a second flavivirus, wherein the transcriptional unit directs the synthesis of the antigen.

2. The nucleic acid of claim 1, wherein the signal sequence is a Japanese encephalitis virus signal sequence.

3. The nucleic acid of claim 1, wherein the immunogenic flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.

4. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of West Nile virus.

5. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of yellow fever virus.

6. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of St. Louis encephalitis virus.

7. The nucleic acid of claim 1, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus and an M protein and an E protein of Powassan virus.

8. The nucleic acid of claim 1, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.

9. The nucleic acid of claim 8, wherein the antigen is both the M protein and the E protein of a flavivirus.

10. The nucleic acid of claim 1, wherein the nucleic acid is DNA.

11. The nucleic acid of claim 10, comprising a nucleotide sequence selected from the group consisting of SEQ ID NO:15, SEQ ID NO:19, SEQ ID NO:21 and SEQ ID NO:23.

12. The nucleic acid of claim 1, wherein the transcriptional unit comprises a control sequence disposed appropriately such that it operably controls the synthesis of the antigen.

13. The nucleic acid of claim 12, wherein the control sequence is the cytomegalovirus immediate early promoter.

14. The nucleic acid of claim 1, comprising a Kozak consensus sequence located at a translational start site for a polypeptide comprising the antigen encoded by the TU.

15. The nucleic acid of claim 1 wherein the transcriptional unit comprises a poly-A terminator.

16. A cell comprising the nucleic acid of claim 1.

17. A composition comprising the nucleic acid of claim 1 and a pharmaceutically acceptable carrier.

18. A method of immunizing a subject against infection by a flavivirus, comprising administering to the subject an effective amount of the

composition of claim 17.

19. The method of claim 18, wherein the flavivirus antigen is of a flavivirus selected from the group consisting of yellow fever virus, dengue serotype 1 virus, dengue serotype 2 virus, dengue serotype 3 virus, dengue serotype 4 virus, Japanese encephalitis virus, Powassan virus and West Nile virus.

20. The method of claim 18, wherein the antigen is selected from the group consisting of an M protein of a flavivirus, an E protein of a flavivirus, both an M protein and an E protein of a flavivirus, a portion of an M protein of a flavivirus, a portion of an E protein of a flavivirus and both a portion of an M protein of a flavivirus and a portion of an E protein of a flavivirus or any combination thereof.

21. The method of claim 20, wherein the antigen is both the M protein and the E protein of a flavivirus, and wherein a cell within the body of the subject, after incorporating the nucleic acid within it, secretes subviral particles comprising the M protein and the E protein.

22. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of West Nile virus.

23. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of yellow fever virus.

24. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of St. Louis encephalitis virus.

25. The method of claim 18, wherein the transcriptional unit encodes a signal sequence of Japanese encephalitis virus, and an M protein and an E protein of Powassan virus.

26. The method of claim 18, comprising administering the composition to the subject in a single dose.

27. The method of claim 18, wherein the composition is administered via a parenteral route.

28. The nucleic acid of claim 1, wherein the antigen is a St. Louis encephalitis virus antigen.

29. The method of claim 18, wherein the antigen is a St. Louis encephalitis virus antigen.

30. The nucleic acid of claim 1, wherein the antigen is a Japanese encephalitis virus antigen.

31. The method of claim 18, wherein the antigen is a Japanese encephalitis virus antigen.

32. The nucleic acid of claim 1, wherein the antigen is a yellow fever virus antigen.

33. The method of claim 18, wherein the antigen is a yellow fever virus antigen.

34. The nucleic acid of claim 1, wherein the antigen is a dengue virus antigen.

35. The method of claim 18, wherein the antigen is a dengue virus antigen.

36. The nucleic acid of claim 1, wherein the antigen is a West Nile virus antigen.

37. The method of claim 18, wherein the antigen is a West Nile virus antigen.

38. An antigen produced from the nucleic acid of claim 1.

39. A method of detecting a flavivirus antibody in a sample, comprising: (a) contacting the sample with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antibody in the sample.

40. An antibody produced in response to immunization by the antigen of claim 38.

41. A method of detecting a flavivirus antigen in a sample, comprising: (a) contacting the sample with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby detecting a flavivirus antigen in a sample.

42. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antigen of claim 38 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby diagnosing a flavivirus infection in a subject.

43. A method of diagnosing a flavivirus infection in a subject, comprising: (a) contacting a sample from the subject with the antibody of claim 40 under conditions whereby an antigen/antibody complex can form; and (b) detecting antigen/antibody complex formation, thereby diagnosing a flavivirus infection in a subject.

=> file wpids

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	5.46	5.88

FILE 'WPIDS' ENTERED AT 16:00:09 ON 30 APR 2004
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FILE LAST UPDATED: 29 APR 2004 <20040429/UP>
MOST RECENT DERWENT UPDATE: 200428 <200428/DW>
DERWENT WORLD PATENTS INDEX SUBSCRIBER FILE, COVERS 1963 TO DATE

>>> FOR A COPY OF THE DERWENT WORLD PATENTS INDEX STN USER GUIDE, PLEASE VISIT:
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>>> THE DISPLAY LAYOUT HAS BEEN CHANGED TO ACCOMODATE THE
NEW FORMAT GERMAN PATENT APPLICATION AND PUBLICATION
NUMBERS. SEE ALSO:
http://www.stn-international.de/archive/stnews/news0104.pdf <<<

>>> SINCE THE FILE HAD NOT BEEN UPDATED BETWEEN APRIL 12-16
THERE WAS NO WEEKLY SDI RUN <<<

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=> e chang g j/in
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E10           1     CHANG G Q/IN
E11          17     CHANG G R/IN
E12         407     CHANG G S/IN

=> s e3
L2          106 "CHANG G J"/IN

=> s l2 and flavivir?
            303 FLAVIVIR?
L3            2 L2 AND FLAVIVIR?

=> d l3,bib,ab,1-2

L3   ANSWER 1 OF 2  WPIDS  COPYRIGHT 2004 THOMSON DERWENT on STN
Full Text
AN   2003-058572 [05]   WPIDS
CR   2000-072885 [06]
DNN  N2003-045382       DNC C2003-015067
TI   Novel isolated nucleic acid useful as vaccine for preventing flavivirus
     infection, comprises transcriptional unit encoding signal sequence of one
     flavivirus and immunogenic flavivirus antigen of a second flavivirus.
DC   B04 D16 S03
IN   CHANG, G J
PA   (USSH) US DEPT HEALTH & HUMAN SERVICES
CYC  101
PI   WO 2002081754   A1 20021017 (200305)* EN  174
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            NL OA PT SD SE SL SZ TR TZ UG ZM ZW
         W: AE AG AL AM AT AU AZ BA BB BG BR BY BZ CA CH CN CO CR CU CZ DE DK
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            KZ LC LK LR LS LT LU LV MA MD MG MK MN MW MX MZ NO NZ OM PH PL PT
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            RO SE SI TR
     BR 2002008301   A  20040309 (200420)
     KR 2003092051   A  20031203 (200424)
ADT  WO 2002081754 A1 WO 2002-US10764 20020404; EP 1383931 A1 EP 2002-763960
     20020404, WO 2002-US10764 20020404; BR 2002008301 A BR 2002-8301 20020404,
     WO 2002-US10764 20020404; KR 2003092051 A KR 2003-713021 20031002
FDT  EP 1383931 A1 Based on WO 2002081754; BR 2002008301 A Based on WO
     2002081754
PRAI US 2001-826115       20010404
```

AB WO 200201754 A UPAB: 20040406

NOVELTY - An isolated nucleic acid (I) comprising a transcriptional unit encoding a signal sequence of a structural protein of a first **flavivirus** and an immunogenic **flavivirus** antigen of a second **flavivirus**, where the transcriptional unit directs the synthesis of the antigen, is new.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for:

(1) a cell (II) comprising (I);

(2) a composition (III) comprising (I) and a pharmaceutically acceptable carrier;

(3) an antigen (IV) produced from (I); and

(4) an antibody (V) produced in response to immunization by (IV).

ACTIVITY - Virucide.

MECHANISM OF ACTION - Vaccine (claimed).

Three-day (mixed sex) or 3-week-old (female) ICR outbred mice, 10 per group, were vaccinated intramuscularly with 50 or 100 mu g of nucleic acid transcriptional unit (TU)-containing vaccine constructs, or subcutaneously with doses of JE-VAX that were one-tenth or one-fifth the dose given to humans. 3-day old vaccinated groups were challenged 7 weeks after vaccination by intraperitoneal injection of 50000 plaque forming units (pfu)/100 mu l of the mouse-adapted Japanese encephalitis virus (JEV) strain SA14 and observed for 3 weeks. 100% protection was achieved in groups that received various nucleic acid TU-containing vaccine constructs for up to 21 days. In contrast, 60% of the JE-VAX-vaccinated mice did not survive virus challenge by 21 days. These results indicated that the nucleic acid TU's of conferred effective protection on vaccinated mice. This suggested the possibility of employing the nucleic acid vaccine as an early childhood vaccine for humans. In contrast, JE-VAX, the inactivated human vaccine currently used, did not appear to be effective in young animals.

USE - (III) is useful for immunizing a subject against infection by a **flavivirus**, by administering an effective amount of (III) to the subject. (IV) is useful for detecting **flavivirus** antibody in a sample, by contacting the sample with (IV) under conditions to form an antigen/antibody complex, and detecting antigen/antibody complex formation, thus detecting a **flavivirus** antibody in the sample. (V) is useful for detecting a **flavivirus** antigen in a sample, by contacting the sample with (V) under conditions to form an antigen/antibody complex, and detecting antigen/antibody complex formation, thus detecting a **flavivirus** antigen in the sample. (IV) or (V) is useful for diagnosing a **flavivirus** infection in a subject, by contacting the sample from the subject with (IV) or (V) under conditions to form an antigen/antibody complex, and detecting antigen/antibody complex formation, therefore diagnosing a **flavivirus** infection in the subject (claimed). (I) is useful as a vaccine for preventing **flavivirus** infection.

ADVANTAGE - (I) is easy to prepare and administer and is stable in storage prior to use. (I) is essentially 100% successful in conferring protective immunity in mammals after administering only a single dose. The nucleic acid transcriptional unit is able to engender immunity to a **flavivirus** in a female mammal which can be transmitted its progeny to the milk.

Dwg.0/8

L3 ANSWER 2 OF 2 WPIDS COPYRIGHT 2004 THOMSON DERWENT on STN
Full Text
AN 2000-072885 [06] WPIDS
CR 2003-058572 [05]
DNC C2000-020998
TI Novel nucleic acid for use in vaccines.
DC B04 D16
IN **CHANG, G J**
PA (USSH) US DEPT HEALTH & HUMAN SERVICES; (USSH) US CENTERS DISEASE CONTROL & PREVENTION; (CHAN-I) CHANG G J
CYC 86
PI WO 9963095 A1 19991209 (200006)* EN 58
RW: AT BE CH CY DE DK EA ES FI FR GB GH GM GR IE IT KE LS LU MC MW NL OA PT SD SE SL SZ UG ZW

W: AE AG AM AT AU AZ BA BB BG BR BY CA CH CN CO CZ DE DK EE ES FI GB
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TT UA UG US UZ VN YU ZA ZW
AU 9943296 A 19991220 (200021)
BR 9910830 A 20010213 (200114)
EP 1084252 A1 20010321 (200117) EN
R: DE FR GB NL
JP 2002517200 W 20020618 (200242) 63
US 2003022849 A1 20030130 (200311)
ADT WO 9963095 A1 WO 1999-US12298 19990603; AU 9943296 A AU 1999-43296 19990603; BR 9910830 A BR 1999-10830 19990603, WO 1999-US12298 19990603; EP 1084252 A1 EP 1999-955295 19990603, WO 1999-US12298 19990603; JP 2002517200 W WO 1999-US12298 19990603, JP 2000-552289 19990603; US 2003022849 A1 Provisional US 1998-87908P 19980604, CIP of WO 1999-US12298 19990603, US 2001-826115 20010404, CIP of US 2001-701536 20010618
FDT AU 9943296 A Based on WO 9963095; BR 9910830 A Based on WO 9963095; EP 1084252 A1 Based on WO 9963095; JP 2002517200 W Based on WO 9963095
PRAI US 1998-87908P 19980604; US 2001-826115 20010404;
US 2001-701536 20010618
AB WO 9963095 A UPAB: 20030214
NOVELTY - Nucleic acid molecule (I) comprises a transcription unit (TU) for an immunogenic **flavivirus** antigen (Ag). When incorporated into a host cell, TU directs synthesis of Ag.

DETAILED DESCRIPTION - INDEPENDENT CLAIMS are also included for the following:

(1) host cells containing (I); and

(2) vaccines containing (I) plus a carrier.

ACTIVITY - Antiviral.

MECHANISM OF ACTION - Vaccine.

USE - (I) are used in vaccines to protect against **flavivirus** infection. Also (not claimed) (I) can be used to produce Ag for analytical or diagnostic applications. Plasmid pCBJE1-14 contains a fragment of nucleic acid encoding the pre-M and E proteins of Japanese encephalitis virus (JEV) cloned into pCBamp. It was administered intramuscularly (50-100 mu g) to 3-day old mice. After 7 weeks all animals were seropositive for JEV and all were protected against subsequent challenge by the mouse-adapted SA14 strain of JEV (contrast 40% survival for animals inoculated with the commercial vaccine JE-VAX).

ADVANTAGE - (I) makes possible inexpensive and safe production of a storage-stable vaccine that has minimal risk of causing adverse immunological reactions to impurities. The vaccines elicit neutralizing antibodies and protective immunity very effectively (i.e. 100% protection), and since they contain only part of the viral genome they can not cause infection in those manufacturing or receiving them. The immunity conferred by the vaccine is transmitted to offspring through the milk.

Dwg.0/8

=> file medline

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	14.34	20.22

FILE 'MEDLINE' ENTERED AT 16:01:13 ON 30 APR 2004

FILE LAST UPDATED: 29 APR 2004 (20040429/UP). FILE COVERS 1951 TO DATE.

On February 29, 2004, the 2004 MeSH terms were loaded. See HELP RLOAD for details. OLDMEDLINE now back to 1951.

MEDLINE thesauri in the /CN, /CT, and /MN fields incorporate the MeSH 2004 vocabulary. See http://www.nlm.nih.gov/mesh/ and http://www.nlm.nih.gov/pubs/techbull/nd03/nd03_mesh.html for a description of changes.

This file contains CAS Registry Numbers for easy and accurate substance identification.

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=> e chang g j/au
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E11         28     CHANG G T/AU
E12          3     CHANG G T G/AU

=> s e3
L4          49 "CHANG G J"/AU

=> s l4 and (flavivir? or yellow or dengue or encephalitis)
          3172 FLAVIVIR?
         18667 YELLOW
          4267 DENGUE
         26248 ENCEPHALITIS
L5           29 L4 AND (FLAVIVIR? OR YELLOW OR DENGUE OR ENCEPHALITIS)

=> s l5 and (prM or E or prM/M or prM/E)
'M' IS NOT A VALID FIELD CODE
'E' IS NOT A VALID FIELD CODE
           544 PRM
        628030 E
             0 PRM/M
             0 PRM/E
L6           9 L5 AND (PRM OR E OR PRM/M OR PRM/E)

=> d l6,cbib,ab,1-9

L6   ANSWER 1 OF 9     MEDLINE on STN
2001200576.    PubMed ID: 11287553.   West Nile virus recombinant DNA vaccine
     protects mouse and horse from virus challenge and expresses in vitro a
     noninfectious recombinant antigen that can be used in enzyme-linked
     immunosorbent assays. Davis B S; Chang G J; Cropp B; Roehrig J T; Martin
     D A; Mitchell C J; Bowen R; Bunning M L. (Division of Vector-Borne
     Infectious Diseases, Centers for Disease Control and Prevention, Public
     Health Service, U.S. Department of Health and Human Services, Fort
     Collins, Colorado 80522, USA. ) Journal of virology, (2001 May) 75 (9)
     4040-7.  Journal code: 0113724. ISSN: 0022-538X. Pub. country: United
     States. Language: English.
AB   Introduction of West Nile (WN) virus into the United States in 1999
     created major human and animal health concerns.  Currently, no human or
     veterinary vaccine is available to prevent WN viral infection, and
     mosquito control is the only practical strategy to combat the spread of
     disease.  Starting with a previously designed eukaryotic expression
     vector, we constructed a recombinant plasmid (pCBWN) that expressed the WN
     virus prM and E proteins.  A single intramuscular injection of pCBWN
     DNA induced protective immunity, preventing WN virus infection in mice and
     horses.  Recombinant plasmid-transformed COS-1 cells expressed and
     secreted high levels of WN virus prM and E proteins into the culture
     medium.  The medium was treated with polyethylene glycol to concentrate
     proteins.  The resultant, containing high-titered recombinant WN virus
     antigen, proved to be an excellent alternative to the more traditional
     suckling-mouse brain WN virus antigen used in the immunoglobulin M (IgM)
     antibody-capture and indirect IgG enzyme-linked immunosorbent assays.
     This recombinant antigen has great potential to become the antigen of
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choice and will facilitate the standardization of reagents and implementation of WN virus surveillance in the United States and elsewhere.

L6 ANSWER 2 OF 9 MEDLINE on STN

2000219418. PubMed ID: 10756038. A single intramuscular injection of recombinant plasmid DNA induces protective immunity and prevents Japanese **encephalitis** in mice. **Chang G J**; Hunt A R; Davis B. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado 80522, USA.. gxc7@cdc.gov) . Journal of virology, (2000 May) 74 (9) 4244-52. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB Plasmid vectors containing Japanese **encephalitis** virus (JEV) premembrane (**prM**) and envelope (**E**) genes were constructed that expressed **prM** and **E** proteins under the control of a cytomegalovirus immediate-early gene promoter. COS-1 cells transformed with this plasmid vector (JE-4B clone) secreted JEV-specific extracellular particles (EPs) into the culture media. Groups of outbred ICR mice were given one or two doses of recombinant plasmid DNA or two doses of the commercial vaccine JEVAX. All mice that received one or two doses of DNA vaccine maintained JEV-specific antibodies 18 months after initial immunization. JEVAX induced 100% seroconversion in 3-week-old mice; however, none of the 3-day-old mice had enzyme-linked immunosorbent assay titers higher than 1:400. Female mice immunized with this DNA vaccine developed plaque reduction neutralization antibody titers of between 1:20 and 1:160 and provided 45 to 100% passive protection to their progeny following intraperitoneal challenge with 5,000 PFU of virulent JEV strain SA14. Seven-week-old adult mice that had received a single dose of JEV DNA vaccine when 3 days of age were completely protected from a 50, 000-PFU JEV intraperitoneal challenge. These results demonstrate that a recombinant plasmid DNA which produced JEV EPs in vitro is an effective vaccine.

L6 ANSWER 3 OF 9 MEDLINE on STN

1998063965. PubMed ID: 9402373. Imported **yellow** fever in a United States citizen. McFarland J M; Baddour L M; Nelson J E; Elkins S K; Craven R B; Cropp B C; **Chang G J**; Grindstaff A D; Craig A S; Smith R J. (Department of Medicine, Graduate School of Medicine, The University of Tennessee Medical Center at Knoxville, 37920-6999, USA.) Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, (1997 Nov) 25 (5) 1143-7. Journal code: 9203213. ISSN: 1058-4838. Pub. country: United States. Language: English.

AB The last imported case of **yellow** fever seen in this country was in 1924. We report a case of **yellow** fever acquired by an American tourist who visited the jungles of Brazil along the Rio Negro and Amazon Rivers. The patient died 6 days after hospital admission and 10 days after his first symptoms appeared. **Yellow** fever virus was recovered from clinical specimens, and the isolate was genetically similar to the **E** genotype IIB of South American **yellow** fever viruses. This patient's illness represents a case of vaccine-preventable death since he failed to be immunized with a recommended preexposure **yellow** fever vaccine.

L6 ANSWER 4 OF 9 MEDLINE on STN

97288308. PubMed ID: 9143286. Construction of infectious cDNA clones for **dengue** 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. Kinney R M; Butrapet S; **Chang G J**; Tsuchiya K R; Roehrig J T; Bhamarapravati N; Gubler D J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.. rmk1@cdc.gov) . Virology, (1997 Apr 14) 230 (2) 300-8. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB We identified nine nucleotide differences between the genomes of **dengue**-2 (DEN-2) 16681 virus and its vaccine derivative, strain PDK-53. These included a C-to-T (16681-to-PDK-53) mutation at nucleotide position 57 of the 5'-untranslated region, three silent mutations, and substitutions **prM**-29 Asp to Val, NS1-53 Gly to Asp, NS2A-181 Leu to Phe,

NS3-250 Glu to Val, and NS4A 75 Gly to Ala. Unpassaged PDK-53 vaccine contained two genetic variants as a result of partial mutation at NS3-250. We constructed infectious cDNA clones for 16681 virus and each of the two PDK-53 variants. DEN-2 16681 clone-derived viruses were identical to the 16681 virus in plaque size and replication in LLC-MK2 cells, replication in C6/36 cells, **E** and **prM** epitopes, and neurovirulence for suckling mice. PDK-53 virus and both clone-derived PDK-53 variants were attenuated in mice. However, the variant containing NS3-250-Glu was less temperature sensitive and replicated better in C6/36 cells than did PDK-53 virus. The variant containing NS3-250-Val had smaller, more diffuse plaques, decreased replication, and increased temperature sensitivity in LLC-MK2 cells relative to PDK-53 virus. Both PDK-53 virus and the NS3-250-Val variant replicated poorly in C6/36 cells relative to 16681 virus. Unpassaged PDK-53 vaccine virus and the virus passaged once in LLC-MK2 cells had genomes of identical sequence, including the mixed NS3-250-Glu/Val locus. Although the NS3-250-Val mutation clearly affected virus replication in vitro, it was not a major determinant of attenuation for PDK-53 virus in suckling mice.

L6 ANSWER 5 OF 9 MEDLINE on STN

95363991. PubMed ID: 7637022. Nucleotide sequence variation of the envelope protein gene identifies two distinct genotypes of **yellow** fever virus. **Chang G J**; Cropp B C; Kinney R M; Trent D W; Gubler D J. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522, USA.) Journal of virology, (1995 Sep) 69 (9) 5773-80. Journal code: 0113724. ISSN: 0022-538X. Pub. country: United States. Language: English.

AB The evolution of **yellow** fever virus over 67 years was investigated by comparing the nucleotide sequences of the envelope (**E**) protein genes of 20 viruses isolated in Africa, the Caribbean, and South America. Uniformly weighted parsimony algorithm analysis defined two major evolutionary **yellow** fever virus lineages designated **E** genotypes I and II. **E** genotype I contained viruses isolated from East and Central Africa. **E** genotype II viruses were divided into two sublineages: IIA viruses from West Africa and IIB viruses from America, except for a 1979 virus isolated from Trinidad (TRINID79A). Unique signature patterns were identified at 111 nucleotide and 12 amino acid positions within the **yellow** fever virus **E** gene by signature pattern analysis. **Yellow** fever viruses from East and Central Africa contained unique signatures at 60 nucleotide and five amino acid positions, those from West Africa contained unique signatures at 25 nucleotide and two amino acid positions, and viruses from America contained such signatures at 30 nucleotide and five amino acid positions in the **E** gene. The dissemination of **yellow** fever viruses from Africa to the Americas is supported by the close genetic relatedness of genotype IIA and IIB viruses and genetic evidence of a possible second introduction of **yellow** fever virus from West Africa, as illustrated by the TRINID79A virus isolate. The **E** protein genes of American IIB **yellow** fever viruses had higher frequencies of amino acid substitutions than did genes of **yellow** fever viruses of genotypes I and IIA on the basis of comparisons with a consensus amino acid sequence for the **yellow** fever **E** gene. The great variation in the **E** proteins of American **yellow** fever virus probably results from positive selection imposed by virus interaction with different species of mosquitoes or nonhuman primates in the Americas.

L6 ANSWER 6 OF 9 MEDLINE on STN

95146982. PubMed ID: 7844560. Molecular basis of attenuation of neurovirulence of wild-type Japanese **encephalitis** virus strain SA14. Ni H; **Chang G J**; Xie H; Trent D W; Barrett A D. (Department of Pathology F-05, University of Texas Medical Branch, Galveston 77555-0605.) Journal of general virology, (1995 Feb) 76 (Pt 2) 409-13. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB To identify the molecular determinants for attenuation of wild-type Japanese **encephalitis** (JE) virus strain SA14, the RNA genome of wild-type strain SA14 and its attenuated vaccine virus SA14-2-8 were reverse transcribed, amplified by PCR and sequenced. Comparison of the

nucleotide sequence of SA14-2-8 vaccine virus with virulent parent SA14 virus and with two other attenuated vaccine viruses derived from SA14 virus (SA14-14-2/PHK and SA14-14-2/PDK) revealed only seven amino acids in the virulent parent SA14 had been substituted in all three attenuated vaccines. Four were in the envelope (**E**) protein (**E**-138, **E**-176, **E**-315 and **E**-439), one in non-structural protein 2B (NS2B-63), one in NS3 (NS3-105), and one in NS4B (NS4B-106). The substitutions at **E**-315 and **E**-439 arose due to correction of the SA14/CDC sequence published previously by Nitayaphan et al. (Virology 177, 541-552, 1990). The mutations in NS2B and NS3 are in functional domains of the trypsin-like serine protease. Attenuation of SA14 virus may therefore, in part, be due to alterations in viral protease activity, which could affect replication of the virus.

L6 ANSWER 7 OF 9 MEDLINE on STN

94267439. PubMed ID: 8207417. Comparison of nucleotide and deduced amino acid sequence of the 5' non-coding region and structural protein genes of the wild-type Japanese **encephalitis** virus strain SA14 and its attenuated vaccine derivatives. Ni H; Burns N J; **Chang G J**; Zhang M J; Wills M R; Trent D W; Sanders P G; Barrett A D. (Department of Pathology F-05, University of Texas Medical Branch, Galveston 77555-0605.) Journal of general virology, (1994 Jun) 75 (Pt 6) 1505-10. Journal code: 0077340. ISSN: 0022-1317. Pub. country: ENGLAND: United Kingdom. Language: English.

AB Nucleotide sequences of the 5' non-coding region and the structural protein genes of the live, attenuated Japanese **encephalitis** vaccine virus strains SA14-2-8 and SA14-5-3 and the wild-type parental strain SA14/USA were determined. SA14-2-8 differed from SA14/USA by 13 nucleotides and eight amino acids whereas SA14-5-3 differed from SA14/USA by 15 nucleotides and eight amino acids. A comparison of the 5' non-coding region and amino acid sequences of the structural proteins of these two attenuated vaccine strains and of vaccine strains SA14-14-2/PHK and SA14-14-2/PDK with three sequences of their wild-type parent SA14 virus was performed. This revealed only two common amino acid substitutions at positions 138 and 176 in the envelope (**E**) protein. The substitution at E138 was predicted to cause a change in the secondary structure of the **E** protein. These two amino acid substitutions in the **E** protein may contribute to attenuation of the Japanese **encephalitis** vaccine viruses.

L6 ANSWER 8 OF 9 MEDLINE on STN

94025568. PubMed ID: 8212556. Phylogenetic relationships of **dengue**-2 viruses. Lewis J A; **Chang G J**; Lanciotti R S; Kinney R M; Mayer L W; Trent D W. (Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colorado 80522.) Virology, (1993 Nov) 197 (1) 216-24. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB RNA oligonucleotide fingerprinting studies on a large number of virus isolates previously demonstrated considerable genetic variation in isolates of **dengue** (DEN)-2 serotype. We report the entire envelope (**E**) glycoprotein gene and deduced amino acid sequences of 16 DEN-2 viruses and the phylogenetic relationships of these, plus 17 additional published DEN **E** gene sequences. Comparison of DEN-2 **E** glycoprotein gene sequences revealed base substitutions scattered throughout the entire gene with as much as 22% sequence divergence. Aligned **E** glycoprotein amino acid sequences revealed the viruses differed by as much as 10%. There appeared to be constraints on the overall structure of the **E** protein to maintain biological function. Clusters of amino acid substitutions were present in the hydrophobic membrane anchor region at the carboxyl terminal end of the protein. Maximum parsimony analysis of the **E** gene sequences allowed construction of a phylogram indicating evolutionary relationships of the virus isolates within the DEN-2 serotype. Five genetic subtypes were identified. Phylogenetic relationships of the DEN-2 serotype and other **flaviviruses** based on **E** protein sequences reflected traditional antigenic and serologic classifications.

L6 ANSWER 5 OF 5 MEDLINE on STN

90320126. PubMed ID: 2371768. Nucleotide sequence of the virulent SA-14 strain of Japanese **encephalitis** virus and its attenuated vaccine derivative, SA-14-14-2. Nitayaphan S; Grant J A; **Chang G J**; Trent D W. (Division of Vector-Borne Infections Diseases, Centers for Disease Control, Fort Collins, Colorado 80522.) Virology, (1990 Aug) 177 (2) 541-52. Journal code: 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The attenuated SA-14-14-2 strain of Japanese encephalitis (JE) **virus** has been used to immunize people in the People's Republic of China. Oligonucleotide fingerprints of the parent SA-14 and vaccine strain indicate that multiple genetic changes occurred during attenuation of the virus. We have cloned and sequenced the genomes of both the virulent SA-14 and attenuated SA-14-14-2 viruses to define molecular differences in the genomes. Forty-five nucleotide differences, resulting in 15 amino acid substitutions, were found by comparing sequences of the SA-14 and SA-14-14-2 genomes. Transversion of U to A occurred at position 39 in the 5'-noncoding region of SA-14-14-2 and another SA-14 vaccine derivative SA-14-5-3. A single nucleotide change in the capsid gene of SA-14-14-2 altered a single amino acid which changed its predicted secondary structure. A silent nucleotide change was found in the **prM** gene sequence and the M-protein was unchanged. There are seven nucleotide differences, resulting in five amino acid changes, in the **E** glycoprotein sequence of the two viruses. Nine amino acid differences were found in the nonstructural proteins of SA-14 and SA-14-14-2: one in NS2A, two in NS2B, three in NS3, one in ns4a, and two in NS5. A single nucleotide change at position 10,428 in the 3'-noncoding region is vaccine virus-specific. The nucleotide and deduced amino acid sequences of the vaccine strain SA-14-14-2, the parent virus SA-14, and virulent strains JaOArS982 and Beijing-1 have been compared and are highly conserved.

=> file uspatful

COST IN U.S. DOLLARS	SINCE FILE ENTRY	TOTAL SESSION
FULL ESTIMATED COST	5.40	25.62

FILE 'USPATFULL' ENTERED AT 16:06:37 ON 30 APR 2004
CA INDEXING COPYRIGHT (C) 2004 AMERICAN CHEMICAL SOCIETY (ACS)

FILE COVERS 1971 TO PATENT PUBLICATION DATE: 29 Apr 2004 (20040429/PD)
FILE LAST UPDATED: 29 Apr 2004 (20040429/ED)
HIGHEST GRANTED PATENT NUMBER: US6728968
HIGHEST APPLICATION PUBLICATION NUMBER: US2004083524
CA INDEXING IS CURRENT THROUGH 29 Apr 2004 (20040429/UPCA)
ISSUE CLASS FIELDS (/INCL) CURRENT THROUGH: 29 Apr 2004 (20040429/PD)
REVISED CLASS FIELDS (/NCL) LAST RELOADED: Feb 2004
USPTO MANUAL OF CLASSIFICATIONS THESAURUS ISSUE DATE: Feb 2004

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>>> original, i.e., the earliest published granted patents or <<<
>>> applications. USPAT2 contains full text of the latest US <<<
>>> publications, starting in 2001, for the inventions covered in <<<
>>> USPATFULL. A USPATFULL record contains not only the original <<<
>>> published document but also a list of any subsequent <<<
>>> publications. The publication number, patent kind code, and <<<
>>> publication date for all the US publications for an invention <<<
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>>> /PK, etc. <<<

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>>> <<<
>>> Use USPATALL when searching terms such as patent assignees, <<<

>>> classifications, or claims, that may potentially change from <<<
>>> the earliest to the latest publication. <<<

This file contains CAS Registry Numbers for easy and accurate
substance identification.

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          1859 FLAVIVIR?
        213719 YELLOW
         16385 FEVER
        583930 VIR?
           772 YELLOW FEVER VIR?
                 (YELLOW(W)FEVER(W)VIR?)
          1636 DENGUE
          5623 ENCEPHALITIS
        583930 VIR?
          1560 ENCEPHALITIS VIR?
                 (ENCEPHALITIS(W)VIR?)
L7        3470 (FLAVIVIR? OR YELLOW FEVER VIR? OR DENGUE OR ENCEPHALITIS VIR?)

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            42 PREMEMBRANE
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=> s l8 and (E or envelope)
       2337562 E
        106147 ENVELOPE
L9            79 L8 AND (E OR ENVELOPE)

=> s l9 and (M or membrane)
       1141222 M
        202153 MEMBRANE
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        626803 SEQUENCE
         21980 SIGNAL SEQUENCE
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L12            5 L11 AND KOZAK

=> d l12,bib,1-5

L12  ANSWER 1 OF 5  USPATFULL on STN
Full Text
AN     2004:12950  USPATFULL
TI     Novel flavivirus antigens
IN     Apt, Doris, Sunnyvale, CA, UNITED STATES
       Punnonen, Juha, Belmont, CA, UNITED STATES
       Brinkman, Alice M., Lake Bluff, IL, UNITED STATES
PA     Maxygen, Inc. Patent Department, Redwood City, CA, UNITED STATES (U.S.
       corporation)
PI     US 2004009469      A1   20040115
AI     US 2003-375932     A1   20030226 (10)
PRAI   US 2002-360030P     20020226 (60)
DT     Utility
FS     APPLICATION
LREP   MAXYGEN, INC., INTELLECTUAL PROPERTY DEPARTMENT, 515 GALVESTON DRIVE,
       RED WOOD CITY, CA, 94063
CLMN   Number of Claims: 117
ECL    Exemplary Claim: 1
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DRWN 16 Drawing Page(s)
LN.CNT 19719
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L12 ANSWER 2 OF 5 USPATFULL on STN
Full Text
AN 2003:330113 USPATFULL
TI Chimeric alphavirus replicon particles
IN Polo, John M., Hayward, CA, UNITED STATES
Perri, Silvia, Castro Valley, CA, UNITED STATES
Thudium, Kent, Oakland, CA, UNITED STATES
Tang, Zequn, San Ramon, CA, UNITED STATES
PA Chiron Corporation (U.S. corporation)
PI US 2003232324 A1 20031218
AI US 2002-310734 A1 20021204 (10)
RLI Continuation-in-part of Ser. No. US 2002-123101, filed on 11 Apr 2002, PENDING
PRAI US 2001-295451P 20010531 (60)
DT Utility
FS APPLICATION
LREP Chiron Corporation, Intellectual Property, P.O. Box 8097, Emeryville, CA, 94662-8097
CLMN Number of Claims: 15
ECL Exemplary Claim: 1
DRWN 15 Drawing Page(s)
LN.CNT 3743
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L12 ANSWER 3 OF 5 USPATFULL on STN
Full Text
AN 2003:213625 USPATFULL
TI Chimeric alphavirus replicon particles
IN Polo, John M., Hayward, CA, UNITED STATES
Perri, Silvia, Castro Valley, CA, UNITED STATES
Thudium, Kent, Oakland, CA, UNITED STATES
PI US 2003148262 A1 20030807
AI US 2002-123101 A1 20020411 (10)
PRAI US 2001-295451P 20010531 (60)
DT Utility
FS APPLICATION
LREP CHIRON CORPORATION, Intellectual Property - R440, P.O. Box 8097, Emeryville, CA, 94662-8097
CLMN Number of Claims: 48
ECL Exemplary Claim: 1
DRWN 13 Drawing Page(s)
LN.CNT 3668
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L12 ANSWER 4 OF 5 USPATFULL on STN
Full Text
AN 2003:51224 USPATFULL
TI Peptide extended glycosylated polypeptides
IN Okkels, Jens Sigurd, Vedbaek, DENMARK
Jensen, Anne Dam, Copenhagen, DENMARK
van den Hazel, Bart, Copenhagen, DENMARK
PI US 2003036181 A1 20030220
AI US 2001-896896 A1 20010629 (9)
PRAI DK 2000-1027 20000630
DK 2000-1092 20000714
WO 2000-DK743 20001229
WO 2001-DK90 20010209
US 2000-217497P 20000711 (60)
US 2000-225558P 20000816 (60)
DT Utility
FS APPLICATION
LREP MAXYGEN, INC., 515 GALVESTON DRIVE, RED WOOD CITY, CA, 94063

CLMN Number of Claims: 57
ECL Exemplary Claim: 1
DRWN 2 Drawing Page(s)
LN.CNT 4732
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L12 ANSWER 5 OF 5 USPATFULL on STN
Full Text
AN 2003:30900 USPATFULL
TI Nucleic acid vaccines for prevention of **flavivirus** infection
IN Chang, Gwong-Jen J., Fort Collins, CO, UNITED STATES
PI US 2003022849 A1 20030130
AI US 2001-826115 A1 20010404 (9)
RLI Continuation-in-part of Ser. No. US 2001-701536, filed on 18 Jun 2001, PENDING A 371 of International Ser. No. WO 1999-US12298, filed on 3 Jun 1999, UNKNOWN
PRAI US 1998-87908P 19980604 (60)
DT Utility
FS APPLICATION
LREP Mary L. Miller, Esq., NEEDLE & ROSENBERG, P.C., The Candler Building, Suite 1200, 127 Peachtree Street, N.E., Atlanta, GA, 30303-1811
CLMN Number of Claims: 43
ECL Exemplary Claim: 1
DRWN 5 Drawing Page(s)
LN.CNT 4194
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

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E CHANG GWONG JEN/IN
L1 1 S E4

FILE 'WPIDS' ENTERED AT 16:00:09 ON 30 APR 2004
E CHANG G J/IN
L2 106 S E3
L3 2 S L2 AND FLAVIVIR?

FILE 'MEDLINE' ENTERED AT 16:01:13 ON 30 APR 2004
E CHANG G J/AU
L4 49 S E3
L5 29 S L4 AND (FLAVIVIR? OR YELLOW OR DENGUE OR ENCEPHALITIS)
L6 9 S L5 AND (PRM OR E OR PRM/M OR PRM/E)

FILE 'USPATFULL' ENTERED AT 16:06:37 ON 30 APR 2004
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L8 79 S L7 AND (PRM OR PREMEMBRANE)
L9 79 S L8 AND (E OR ENVELOPE)
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L12 5 S L11 AND KOZAK

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2806970 AY<1999
L14 15 L13 AND AY<1999

=> d l14,cbib,1-15

L14 ANSWER 1 OF 15 USPATFULL on STN
2003:169096 Nucleic acid sequences and expression system relating to

Enterococcus faecium for diagnostics and therapeutics.
Doucette-Stamm, Lynn A., Framingham, MA, United States
Bush, David, Somerville, MA, United States
Genome Therapeutics Corporation, Waltham, MA, United States (U.S. corporation)
US 6583275 B1 20030624
APPLICATION: US 1998-107532 19980630 (9) <--
PRIORITY: US 1998-85598P 19980514 (60)
US 1997-51571P 19970702 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 2 OF 15 USPATFULL on STN
2002:246731 **Dengue** nucleic acid vaccines that induce neutralizing antibodies.
Kochel, Tadeusz J., Frederick, MD, United States
Porter, Kevin R., Gaithersburg, MD, United States
Raviprakash, Kanakatte, Silver Spring, MD, United States
Hoffman, Stephen L., Gaithersburg, MD, United States
Hayes, Curtis G., Frederick, MD, United States
The United States of America as represented by the Secretary of the Navy, Washington, DC, United States (U.S. government)
US 6455509 B1 20020924
APPLICATION: US 1997-869423 19970604 (8) <--
PRIORITY: US 1996-17839P 19960604 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 3 OF 15 USPATFULL on STN
2002:167888 Recombinant nonstructural protein subunit vaccine against **flaviviral** infection.
McDonell, Michael, Kailua, HI, United States
Peters, Iain, Honolulu, HI, United States
Coller, Beth-Ann, Aiea, HI, United States
Hawaii Biotechnology Group, Inc., Aeia, HI, United States (U.S. corporation)
US 6416763 B1 20020709
APPLICATION: US 1998-143077 19980828 (9) <--
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 4 OF 15 USPATFULL on STN
2001:14256 Two-step immunization procedure against the pyramyxoviridae family of viruses using recombinant virus and subunit protein preparation.
Klein, Michel H., Willowdale, Canada
Tartaglia, James, Schenectady, NY, United States
Cates, George A., Richmond Hill, Canada
Ewasyshyn, Mary E., Willowdale, Canada
Virogeneitics Corporation, Troy, NY, United States (U.S. corporation)Connaught Laboratories Limited, North York, Canada (non-U.S. corporation)
US 6180398 B1 20010130
APPLICATION: US 1996-679065 19960712 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 5 OF 15 USPATFULL on STN
2000:174106 Subunit immonogenic composition against **dengue** infection.
Ivy, John, Kailua, HI, United States
Nakano, Eilen, Hon., HI, United States
Clements, David, Honolulu, HI, United States
Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)
US 6165477 20001226
APPLICATION: US 1997-915152 19970820 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 6 OF 15 USPATFULL on STN
2000:142128 Methods of preparing carboxy-terminally truncated recombinant **flavivirus envelope** glycoproteins employing drosophila melanogaster expression systems.
Ivy, John, Kailua, HI, United States
Nakano, Eilen, Honolulu, HI, United States
Clements, David, Honolulu, HI, United States
Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)
US 6136561 20001024
APPLICATION: US 1997-937195 19970925 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 7 OF 15 USPATFULL on STN
2000:74131 Recombinant **dengue** virus DNA fragment.
Kelly, Eileen P., Takoma Park, MD, United States
King, Alan D., Washington, DC, United States
The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 6074865 20000613
APPLICATION: US 1995-504878 19950720 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 8 OF 15 USPATFULL on STN
2000:9526 cDNA sequence of **Dengue** virus serotype 1 (Singapore strain).
Fu, Jianlin, Singapore, Singapore
Tan, Boon-Huan, Singapore, Singapore
Yap, Eu-Hian, Singapore, Singapore
Chan, Yow-Cheong, Singapore, Singapore
Tan, Yin-Hwee, Singapore, Singapore
Insititute of Molecular and Cell Biology, Singapore (non-U.S. corporation)
US 6017535 20000125
WO 9322440 19931111
APPLICATION: US 1994-325426 19941216 (8) <--
WO 1993-CA182 19930428 19941216 PCT 371 date 19941216 PCT 102(e) date
PRIORITY: GB 1992-9243 19920429
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 9 OF 15 USPATFULL on STN
1998:68530 Trova fowl pox virus recombinants comprising heterologous inserts.
Paoletti, Enzo, Delmar, NY, United States
Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States
Norton, Elizabeth K., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyons, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe Francis, Albany, NY, United States
Gettig, Russell Robert, Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5766599 19980616
APPLICATION: US 1995-458101 19950601 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 10 OF 15 USPATFULL on STN
1998:64734 Modified recombinant vaccinia virus and expression vectors thereof.
Paoletti, Enzo, Delmar, NY, United States

Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States
Norton, Elizabeth K., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyon, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe Francis, Albany, NY, United States
Gettig, Russell Robert, Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5762938 19980609
APPLICATION: US 1996-709209 19960821 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 11 OF 15 USPATFULL on STN
1998:57530 Alvac canarypox virus recombinants comprising heterlogous inserts.
Paoletti, Enzo, Delmar, NY, United States
Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States
Norton, Elizabeth K., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyons, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe Francis, Albany, NY, United States
Gettig, Russell Robert, Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5756103 19980526
APPLICATION: US 1995-457007 19950601 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 12 OF 15 USPATFULL on STN
1998:44886 **Flavivirus** recombinant poxvirus immunological composition.
Paoletti, Enzo, Delmar, NY, United States
Pincus, Steven Elliot, East Greenbush, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5744141 19980428
APPLICATION: US 1995-484304 19950607 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 13 OF 15 USPATFULL on STN
1998:44885 **Flavivirus** recombinant poxvirus vaccine.
Paoletti, Enzo, Delmar, NY, United States
Pincus, Steven Elliot, East Greenbush, NY, United States
Virgenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5744140 19980428
APPLICATION: US 1994-224391 19940407 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 14 OF 15 USPATFULL on STN
96:38606 **Flavivirus** recombinant poxvirus vaccine.
Paoletti, Enzo, Delmar, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5514375 19960507
APPLICATION: US 1991-714687 19910613 (7) <--

DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

L14 ANSWER 15 OF 15 USPATFULL on STN
96:16887 NYVAC vaccinia virus recombinants comprising heterologous inserts.
Paoletti, Enzo, Delmar, NY, United States
Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States
Norton, Elizabeth K., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyons, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe F., Albany, NY, United States
Gettig, Russell R., Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5494807 19960227
APPLICATION: US 1993-105483 19930812 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

=> d l14,cbib,ab,clm,kwic

L14 ANSWER 1 OF 15 USPATFULL on STN
2003:169096 Nucleic acid sequences and expression system relating to Enterococcus faecium for diagnostics and therapeutics.
Doucette-Stamm, Lynn A., Framingham, MA, United States
Bush, David, Somerville, MA, United States
Genome Therapeutics Corporation, Waltham, MA, United States (U.S. corporation)
US 6583275 B1 20030624
APPLICATION: US 1998-107532 19980630 (9) <--
PRIORITY: US 1998-85598P 19980514 (60)
US 1997-51571P 19970702 (60)
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The invention provides isolated polypeptide and nucleic acid sequences derived Enterococcus faecium that are useful in diagnosis and therapy of pathological conditions; antibodies against the polypeptides; and methods for the production of the polypeptides. The invention also provides methods for the detection, prevention and treatment of pathological conditions resulting from bacterial infection.

CLM What is claimed is:

1. An isolated nucleic acid consisting of a nucleotide sequence encoding an **E**. faecium polypeptide selected from the group consisting of SEQ ID NO: 3857, SEQ ID NO: 4234, SEQ ID NO: 4304, SEQ ID NO: 4368, SEQ ID NO: 5256, SEQ ID NO: 5965, SEQ ID NO: 5985, SEQ ID NO: 6156, SEQ ID NO: 6320, and SEQ ID NO: 7224.

2. A recombinant expression vector comprising the nucleic acid of claim 1 operably linked to a transcription regulatory element.

3. A cell comprising a recombinant expression vector of claim 2.

4. A single-stranded probe comprising a nucleotide sequence of at least 40 sequential nucleotides selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570.

5. An isolated nucleic acid or the complement thereof consisting of at least 30 consecutive nucleotides of a sequence selected from the group

consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570, wherein the isolated nucleic acid is hybridizable under conditions of high stringency to a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570; wherein the complement indicates full complementarity and of the same length.

6. An isolated nucleic acid selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570.

7. A recombinant expression vector comprising the nucleic acid of claim 6 operably linked to a transcription regulatory element.

8. A cell comprising a recombinant expression vector of claim 7.

9. An isolated nucleic acid consisting of a nucleotide sequence encoding an **E.** faecium polypeptide wherein said isolated nucleic acid consists of at least 40 sequential nucleotides selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570.

10. A recombinant expression vector comprising the nucleic acid of claim 9 operably linked to a transcription regulatory element.

11. A cell comprising a recombinant expression vector of claim 10.

12. A single-stranded probe consisting of a nucleotide sequence consisting of at least 40 sequential nucleotides selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570.

13. An isolated nucleic acid or the complement thereof consisting of at least 20 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570, wherein the isolated nucleic acid is hybridizable under conditions of high stringency to a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570; wherein the complement indicates full complementarity and of the same length.

14. An isolated nucleic acid or the complement thereof consisting of at least 30 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570, wherein the isolated nucleic acid has at least 80% sequence identity with a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and complements thereof; wherein the complement or complements indicates full complementarity and of the same length.

15. An isolated nucleic acid or the complement thereof according to claim 14 wherein the isolated nucleic acid has at least 90% sequence identity with a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID

NO: 2666, SEQ ID NO: 3570 and complements thereof wherein, the complement or complements indicates full complementarity and of the same length.

16. An isolated nucleic acid or the complement thereof consisting of at least 30 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570, wherein the isolated nucleic acid has at least 70% sequence identity with a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, SEQ ID NO: 3570 and complements thereof wherein the complement or complements indicates full complementarity and of the same length.

17. An isolated nucleic acid or the complement thereof consisting of at least 20 consecutive nucleotides of a sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570, wherein the isolated nucleic acid has at least 90% sequence identity with a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2502, SEQ ID NO: 2666, SEQ ID NO: 3570 and complements thereof; wherein the complement or complements indicates full complementarity and of the same length.

18. An isolated nucleic acid consisting of a nucleotide sequence with at least 70% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said isolated nucleic acid encodes an **E.** faecium polypeptide.

19. A recombinant expression vector comprising the nucleic acid of claim 18 operably linked to a transcription regulatory element.

20. A cell comprising a recombinant expression vector of claim 19.

21. An isolated nucleic acid consisting of a nucleotide sequence with at least 80% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said isolated nucleic acid encodes an **E.** faecium polypeptide selected from the group consisting of SEQ ID NO: 3857, SEQ ID NO: 4234, SEQ ID NO: 4304, SEQ ID NO: 4368, SEQ ID NO: 5256, SEQ ID NO: 5965, SEQ ID NO: 5985, SEQ ID NO: 6156, SEQ ID NO: 6320, and SEQ ID NO: 7224, respectively.

22. A recombinant expression vector comprising the nucleic acid of claim 21 operably linked to a transcription regulatory element.

23. A cell comprising a recombinant expression vector of claim 22.

24. An isolated nucleic acid consisting of a nucleotide sequence with at least 90% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 203, SEQ ID NO: 580, SEQ ID NO: 650, SEQ ID NO: 714, SEQ ID NO: 1602, SEQ ID NO: 2311, SEQ ID NO: 2331, SEQ ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said isolated nucleic acid encodes an **E.** faecium polypeptide selected from the group consisting of SEQ ID NO: 3857, SEQ ID NO: 4234, SEQ ID NO: 4304, SEQ ID NO: 4368, SEQ ID NO: 5256, SEQ ID NO: 5965, SEQ ID NO: 5985, SEQ ID NO: 6156, SEQ ID NO: 6320, and SEQ ID NO: 7224, respectively.

25. A recombinant expression vector comprising the nucleic acid of claim 24 operably linked to a transcription regulatory element.

26. A cell comprising a recombinant expression vector of claim 25.

27. A single-stranded probe comprising a nucleotide sequence of at least 30 sequential nucleotides selected from the group consisting of SEQ ID NO: 1602 and SEQ ID NO: 2311.

28. An isolated nucleic acid consisting of a nucleotide sequence encoding an **E**. faecium polypeptide wherein said isolated nucleic acid consists of at least 30 sequential nucleotides selected from the group consisting of SEQ ID NO: 1602 and SEQ ID NO: 2311.

29. A recombinant expression vector comprising the nucleic acid of claim 28 operably linked to a transcription regulatory element.

30. A cell comprising a recombinant expression vector of claim 29.

31. An isolated nucleic acid consisting of a nucleic acid encoding an **E**. faecium polypeptide wherein said isolated nucleic acid consists of at least 20 sequential nucleotides selected from the group consisting of SEQ ID NO: 1602 and SEQ ID NO: 2311.

32. A recombinant expression vector comprising the nucleic acid of claim 31 operably linked to a transcription regulatory element.

33. A cell comprising a recombinant expression vector of claim 32.

34. A single-stranded probe consisting of a nucleotide sequence consisting of at least 20 sequential nucleotides selected from the group consisting of SEQ ID NO: 1602 and SEQ ID NO: 2311.

AI US 1998-107532 19980630 (9) <--

SUMM . . . animals, and humans. Enterococci are part of the normal gastrointestinal and genital tract flora, and among the 17 known species, **E**. faecium is one of the most prominent in humans, with the highest levels of multidrug resistance (A. Kaufhold and R. Klein (1995) Zentralblatt fuer Bakteriologie 282 507). Pathogenic **E**. faecium infections include urinary tract infections (UTI), bacteremia, endocarditis, and wound and abdominal-pelvic infections.

SUMM . . . being combinations of antimicrobials or the use of new unproven compounds (R. C. Moellering, Jr.,1991, J Antimicrob Chemother 28:1; and **M**. K. Hayden et al., 1994, Antimicrob Agents Chemother 38:1225; and N. Mobarakai et al., 1994, J Antimicrob Chemother 33:319). From. . .

SUMM Epidemiology of **E**. faecium is not completely understood, but it is thought that most infections and colonizations are a result of the patient's endogenous flora (B **E** Murray, 1990, Clin Microbiol Rev 3:46). Recent evidence suggests that **E**. faecium can be spread by direct contact with other infected patients, indirect transmission from hospital personnel (J **M** Boyce et al., 1 994, J Clin Microbiol 32:1148-; and **E**. Rhineheart et al., 1990, N Engl J Med 323:1814), or from contaminated hospital surfaces and equipment (L V Karanfil et al., 1992, Infect Control Hosp Epidemiol 13:195; and J **M** Boyce et al., 1994, J Clin Microbiol 32:1148; and L L Livornese Jr., 1992, Ann Intern Med 117:112). Increased risk for the critically ill, those with underlying disease or immunosuppression i.**e**. ICU, oncology, and transplant patients, cardio-thoracic/intraabdominal surgical patients and those with urinary or central venous catheters, has been demonstrated. In addition, risk for; **E**. faecium infection increases for patients with long hospital stays or previous multiantimicrobial or vancomycin treatments (J. **M**. Boyce et al., 1994, J Clin Microbiol 32:1148; Boyle, J. F. et al., 1993, J Clin Microbiol 31:1280; L V Karanfil et al., 1992, Infect Control Hosp Epidemiol 13:195; S. Handwerger et al., 1993, Clin Infect Dis 16:750; Montecalvo, **M**. A. et al., 1994, Antimicrob Agents Chemother 38:1363-1367).

SUMM Additional concern stems from the ability of the **E**. faecium plasmid borne VanA gene, which confers high level vancomycin resistance, to transfer in vitro to several gram positive microorganisms. . . shown

vancomycin resistance conferred by plasmid transfer, but clinically isolated strains of S. haemolyticus have shown vancomycin resistance (Degner, J. **E**. et al., 1994, J Clin Microbiol 32:2260; and Veach, L. A. et al., 1990, J Clin Microbiol 28:2064).

SUMM These concerns point to the need for diagnostic tools and therapeutics aimed at proper identification of **E**. Faecium strains and the eradication of virulence. The design of vaccines that will limit the spread of infection and halt. . .

SUMM . . . for diagnostic tools and therapeutics by providing bacterial-specific compositions and methods for detecting, treating, and preventing bacterial infection, in particular **E**. Faecium infection.

SUMM The present invention encompasses isolated polypeptides and nucleic acids derived from **E**. faecium that are useful as reagents for diagnosis of bacterial infection, components of effective antibacterial vaccines, and/or as targets for antibacterial drugs, including anti-**E**. faecium drugs. The nucleic acids and peptides of the present invention also have utility for diagnostics and therapeutics for **E**. faecium and other Enterococcus species. They can also be used to detect the presence of **E**. faecium and other Enterococcus species in a sample; and in screening compounds for the, ability to interfere with the **E**. faecium life cycle or to inhibit **E**. faecium infection. More specifically, this invention features compositions of nucleic acids corresponding to entire coding sequences of **E**. faecium proteins, including surface or secreted proteins or parts thereof, nucleic acids capable of binding mRNA from **E**. faecium proteins to block protein translation, and methods for producing **E**. faecium proteins or parts thereof using peptide synthesis and recombinant DNA techniques. This invention also features antibodies and nucleic acids useful as probes to detect **E**. faecium infection. In addition, vaccine compositions and methods for the protection or treatment of infection by **E**. faecium are within the scope of this invention.

SUMM . . . refers to a manufacture, other than an isolated nucleic acid molecule, which contains a nucleotide sequence of the present invention, i.**e**., the nucleotide sequence provided in SEQ ID NO: 1-SEQ ID NO: 3654, a fragment thereof, or a nucleotide sequence at. . . Uses for and methods for providing nucleotide sequences in a variety of media is well known in the art (see **e**.g., EPO Publication No. EP 0 756 006)

SUMM . . . Oracle, or the like. A person skilled in the art can readily adapt any number of data processor structuring formats (**e**.g. text file or database) in order to obtain computer readable media having recorded thereon the nucleotide sequence information of the. . .

SUMM Computer algorithms enable the identification of **E**. faecium open reading frames (ORFs) within SEQ ID NO: 1-SEQ ID NO: 3654 which contain homology to ORFs or proteins. . . These algorithms are utilized on computer systems as exemplified below. The ORFs so identified represent protein encoding fragments within the **E**. faecium genome and are useful in producing commercially important proteins such as enzymes used in fermentation reactions and in the. . .

SUMM . . . computer-based systems, which contain the sequence information described herein. Such systems are designed to identify commercially important fragments of the **E**. faecium genome. As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used. . .

SUMM . . . the sequence information stored within the data storage means. Search means are used to identify fragments or regions of the **E**. faecium genome which are similar to, or "match", a particular target sequence or target motif. A variety of known algorithms. . .

SUMM . . . many genes are longer than 500 amino acids, or 1.5 kb in length, and that commercially important fragments of the **E**. faecium genome, such as sequence fragments involved in gene expression and protein processing, will often be shorter than 30 nucleotides.

SUMM . . . variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzymatic active sites, **membrane** spanning regions, and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and. . .

SUMM . . . information in the computer based systems of the present invention. A preferred format for an output means ranks fragments of the **E**. faecium genome possessing varying degrees of homology to the target sequence or target motif. Such presentation provides a person skilled. . .

SUMM . . . used to compare a target sequence or target motif with the data storage means to identify sequence fragments of the **E**. faecium genome. In the present examples, implementing software which implement the BLASTP2 and bic_SW algorithms (Altschul et al., J Mol. Biol. 215:403-410 (1990); Compugen Biocellerator) was used to identify open reading frames within the **E**. faecium genome. A person skilled in the art can readily recognize that any one of the publicly available homology search. . .

SUMM The invention features **E**. faecium polypeptides, preferably a substantially pure preparation of an **E**. faecium polypeptide, or a recombinant **E**. faecium polypeptide. In preferred embodiments: the polypeptide has biological activity; the polypeptide has an amino acid sequence at least 60%,. . . another preferred embodiment, the amino acid sequence which differs in sequence identity by about 7% to about 8% from the **E**. faecium amino acid sequences of the invention contained in the Sequence Listing is also encompassed by the invention.

SUMM In preferred embodiments: the **E**. faecium polypeptide is encoded by a nucleic acid of the invention contained in the Sequence Listing, or by a nucleic. . .

SUMM In a preferred embodiment, the subject **E**. faecium polypeptide differs in amino acid sequence at 1, 2, 3, 5, 10 or more residues from a sequence of the invention contained in the Sequence Listing. The differences, however, are such that the **E**. faecium polypeptide exhibits an **E**. faecium biological activity, **e**.g., the **E**. faecium polypeptide retains a biological activity of a naturally occurring **E**. faecium enzyme.

SUMM In yet other preferred embodiments, the **E**. faecium polypeptide is a recombinant fusion protein having a first **E**. faecium polypeptide portion and a second polypeptide portion, **e**.g., a second polypeptide portion having an amino acid sequence unrelated to **E**. faecium. The second polypeptide portion can be, **e**.g., any of glutathione-S-transferase, a DNA binding domain, or a polymerase activating domain. In preferred embodiment the fusion protein can be. . .

SUMM In a preferred embodiment, the encoded **E**. faecium polypeptide differs (**e**.g., by amino acid substitution, addition or deletion of at least one amino acid residue) in amino acid sequence at 1,. . . more residues, from a sequence of the invention contained in the Sequence Listing. The differences, however, are such that: the **E**. faecium encoded polypeptide exhibits a **E**. faecium biological activity, **e**.g., the encoded **E**. faecium enzyme retains a biological activity of a naturally occurring **E**. faecium.

SUMM The **E**. faecium strain, Mu, from which genomic sequences have been sequenced, has been deposited on Jun. 26, 1997 in the American. . .

SUMM . . . Biology, John Wiley & Sons, New York, 1989, 6.3.1-6.3.6, hereby incorporated by reference); and, polypeptides specifically bound by antisera to **E**. faecium polypeptides, especially by antisera to an active site or binding domain of **E**. faecium polypeptide. The invention also includes fragments, preferably biologically active fragments. These and other polypeptides are also referred to herein as **E**. faecium polypeptide analogs or variants.

SUMM The invention further provides nucleic acids, **e**.g., RNA or DNA, encoding a polypeptide of the invention. This includes double stranded nucleic acids as well as coding and. . .

SUMM In preferred embodiments, the subject **E**. faecium nucleic acid will include a transcriptional regulatory sequence, **e**.g. at least one of a transcriptional promoter or transcriptional enhancer sequence, operably linked to the **E**. faecium gene sequence, **e**.g., to render the **E**. Faecium gene sequence suitable for expression in a recombinant host cell.

SUMM In yet a further preferred embodiment, the nucleic acid which encodes an **E**. faecium polypeptide of the invention, hybridizes under stringent conditions to a nucleic acid probe corresponding to at least 8

consecutive. . .

SUMM In another aspect, the invention provides a substantially pure nucleic acid having a nucleotide sequence which encodes an **E**. faecium polypeptide. In preferred embodiments: the encoded polypeptide has biological activity; the encoded polypeptide has an amino acid sequence at. . .

SUMM In another aspect, the invention encompasses: a vector including a nucleic acid which encodes an **E**. faecium polypeptide or an **E**. faecium polypeptide variant as described herein; a host cell transfected with the vector, and a method of producing a recombinant **E**. faecium polypeptide or **E**. faecium polypeptide variant; including culturing the cell, **e**.g., in a cell culture medium, and isolating an **E**. faecium or **E**. faecium polypeptide variant, **e**.g., from the cell or from the cell culture medium.

SUMM In another aspect, the invention features nucleic acids capable of binding mRNA of **E**. faecium. Such nucleic acid is capable of acting as antisense nucleic acid to control the translation of mRNA of **E**. faecium. A further aspect features a nucleic acid which is capable of binding specifically to an **E**. faecium nucleic acid. These nucleic acids are also referred to herein as complements and have utility as probes and as. . .

SUMM In another aspect, the invention features an expression system comprising an open reading frame corresponding to **E**. faecium nucleic acid. The nucleic acid further comprises a control sequence compatible with an intended host. The expression system is useful for making polypeptides corresponding to **E**. faecium nucleic acid.

SUMM In another aspect, the invention features a cell transformed with the expression system to produce **E**. faecium polypeptides.

SUMM In yet another embodiment, the invention encompasses reagents for detecting bacterial infection, including **E**. faecium infection, which comprise at least one **E**. faecium-derived nucleic acid defined by any one of SEQ ID NO: 1-SEQ ID NO: 3654, or sequence-conservative or function-conservative variants. . .

SUMM . . . for producing antibodies in a host animal. The methods of the invention comprise immunizing an animal with at least one **E**. faecium-derived immunogenic component, wherein the immunogenic component comprises one or more of the polypeptides encoded by any one of SEQ. . . without limitation mammals and birds. Such antibodies have utility as reagents for immunoassays to evaluate the abundance and distribution of **E**. faecium-specific antigens.

SUMM . . . for detecting antibacterial-specific antibodies in a sample, which comprises: (i) contacting a sample suspected to contain antibacterial-specific antibodies with a **E**. faecium antigenic component, under conditions in which a stable antigen-antibody complex can form between the **E**. faecium antigenic component and antibacterial antibodies in the sample; and (ii) detecting any antigen-antibody complex formed in step (i), wherein. . .

SUMM In another aspect, the invention features a method of generating vaccines for immunizing an individual against **E**. faecium. The method includes: immunizing a subject with an **E**. faecium polypeptide, **e**.g., a surface or secreted polypeptide, or active portion thereof, and a pharmaceutically acceptable carrier. Such vaccines have therapeutic and prophylactic. . .

SUMM In another aspect, the invention features a method of evaluating a compound, **e**.g. a polypeptide, **e**.g., a fragment of a host cell polypeptide, for the ability to bind an **E**. faecium polypeptide. The method includes: contacting the candidate compound with an **E**. faecium polypeptide and determining if the compound binds or otherwise interacts with an **E**. faecium polypeptide. Compounds which bind **E**. faecium are candidates as activators or inhibitors of the bacterial life cycle. These assays can be performed in vitro or. . .

SUMM In another aspect, the invention features a method of evaluating a compound, **e**.g. a polypeptide, **e**.g., a fragment of a host cell polypeptide, for the ability to bind an **E**. faecium nucleic acid, **e**.g., DNA or RNA. The method includes: contacting the candidate compound with an **E**. faecium nucleic acid and determining if the

compound binds or otherwise interacts with an **E.** faecium polypeptide. Compounds which bind **E.** faecium are candidates as activators or inhibitors of the bacterial life cycle. These assays can be performed in vitro or. . .

SUMM . . . anti-bacterial candidates. In one embodiment, the target sequence selected is specific to a single species, or even a single strain, i.**e.**, the **E.** faecium Mu. In a second embodiment, the target sequence is common to at least two species of bacteria. In a. . .

SUMM The invention also provides methods for preventing or treating disease caused by certain bacteraii, including **E.** faecium, which are carried out by administering to an animal in need of such treatment, in particular a warm-blooded vertebrate,. . .

SUMM . . . and pyrimidine-containing polymers of any length, either polyribonucleotides or polydeoxyribonucleotides or mixed polyribo-polydeoxyribo nucleotides. This includes single- and double-stranded molecules, i.**e.**, DNA-DNA, DNA-RNA and RNA-RNA hybrids, as well as "protein nucleic acids" (PNA) formed by conjugating bases to an amino acid. . .

SUMM An "**E.** faecium-derived" nucleic acid or polypeptide sequence may or may not be present in other bacterial species, and may or may not be present in all **E.** faecium strains. This term is intended to refer to the source from which the sequence was originally isolated. Thus, a **E.** faecium-derived polypeptide, as used herein, may be used, **e**.g., as a target to screen for a broad spectrum antibacterial agent, to search for homologous proteins in other species of. . .

SUMM . . . from other proteins, lipids, and nucleic acids with which it naturally occurs. Preferably, the polypeptide is also separated from substances, **e**.g., antibodies or gel matrix, **e**.g., polyacrylamide, which are used to purify it. Preferably, the polypeptide constitutes at least 10, 20, 50 70, 80 or 95%. . .

SUMM A purified or isolated or a substantially pure nucleic acid, **e**.g., a substantially pure DNA, (are terms used interchangeably herein) is a nucleic acid which is one or both of the following: not immediately contiguous with both of the coding sequences with which it is immediately contiguous (i.**e.**, one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which. . . which the nucleic acid is derived. The term includes, for example, a recombinant DNA which is incorporated into a vector, **e**.g., into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (**e**.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other DNA sequences. Substantially pure DNA also includes a recombinant DNA which is part of a hybrid gene encoding additional **E.** faecium DNA sequence.

SUMM . . . a position in both of the two compared sequences is occupied by the same base or amino acid monomer subunit, **e**.g., if a position in each of two DNA molecules is occupied by adenine, then the molecules are homologous at that. . .

SUMM . . . (such as, for example 2×SSC at 55° C.), require correspondingly less overall complementarity between the hybridizing sequences. (1×SSC is 0.15 **M** NaCl, 0.015 **M** Na citrate).

SUMM As used herein, the term "surface protein" refers to all surface accessible proteins, **e**.g. inner and outer **membrane** proteins, proteins adhering to the cell wall, and secreted proteins.

SUMM A polypeptide has **E.** faecium biological activity if it has one, two and preferably more of the following properties: (1) if when expressed in the course of an **E.** faecium infection, it can promote, or mediate the attachment of **E.** faecium to a cell; (2) it has an enzymatic activity, structural or regulatory function characteristic of an **E.** faecium protein; (3) or the gene which encodes it can rescue a lethal mutation in an **E.** faecium gene. A polypeptide has biological activity if it is an antagonist, agonist, or super-agonist of a polypeptide having one. . .

SUMM . . . biologically active fragment or analog is one having an in vivo or in vitro activity which is characteristic of the **E.** faecium polypeptides of the invention contained in the Sequence Listing, or of

other naturally occurring **E.** faecium polypeptides, **e.g.**, one or more of the biological activities described herein. Especially preferred are fragments which exist in vivo, **e.g.**, fragments which arise from post transcriptional processing or which arise from translation of alternatively spliced RNA's. Fragments include those expressed in native or endogenous cells as well as those made in expression systems, **e.g.**, in CHO (Chinese Hamster Ovary) cells. Because peptides such as **E.** faecium polypeptides often exhibit a range of physiological properties and because such properties may be attributable to different portions of the molecule, a useful **E.** faecium fragment or **E.** faecium analog is one which exhibits a biological activity in any biological assay for **E.** faecium activity. Most preferably the fragment or analog possesses 10%, preferably 40%, more preferably 60%, 70%, 80% or 90% or greater of the activity of **E.** faecium, in any in vivo or in vitro assay.

SUMM Analogs can differ from naturally occurring **E.** faecium polypeptides in amino acid sequence or in ways that do not involve sequence, or both. Non-sequence modifications include changes in acetylation, methylation, phosphorylation, carboxylation, or glycosylation. Preferred analogs include **E.** faecium polypeptides (or biologically active fragments thereof) whose sequences differ from the wild-type sequence by one or more conservative amino. . . one or more non-conservative amino acid substitutions, deletions, or insertions which do not substantially diminish the biological activity of the **E.** faecium polypeptide. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, **e.g.**, substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine;. . .

SUMM . . . Glu, D-Glu, Gln, D-Gln
Cysteine C D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine Q D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid **E** D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine G Ala, D-Ala, Pro, D-Pro, β-Ala, Acp
Isoleucine I D-Ile, Val, D-Val, Leu, D-Leu,. . . L D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine K D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine **M** D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine F D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline,
. . .

SUMM . . . the peptide bonds) in the peptide sequence. Also included are: analogs that include residues other than naturally occurring L-amino acids, **e.g.**, D-amino acids or non-naturally occurring or synthetic amino acids, **e.g.**, β or γ amino acids; and cyclic analogs.

SUMM As used herein, the term "fragment", as applied to an **E.** faecium analog, will ordinarily be at least about 20 residues, more typically at least about 40 residues, preferably at least about 60 residues in length. Fragments of **E.** faecium polypeptides can be generated by methods known to those skilled in the art. The ability of a candidate fragment to exhibit a biological activity of **E.** faecium polypeptide can be assessed by methods known to those skilled in the art as described herein. Also included are **E.** faecium polypeptides containing residues that are not required for biological activity of the peptide or that result from alternative mRNA. . .

SUMM An "immunogenic component" as used herein is a moiety, such as an **E.** faecium polypeptide, analog or fragment thereof, that is capable of eliciting a humoral and/or cellular immune response in a host. . .

SUMM An "antigenic component" as used herein is a moiety, such as an **E.** faecium polypeptide, analog or fragment thereof, that is capable of binding to a specific antibody with sufficiently high affinity to. . .

SUMM The term "antibody" as used herein is intended to include fragments thereof which are specifically reactive with **E.** faecium polypeptides.

SUMM As used herein, the term "cell-specific promoter" means a DNA sequence that serves as a promoter, i.**e.**, regulates expression of a selected

DNA sequence operably linked to the promoter, and which effects expression of the selected DNA. . .

SUMM Misexpression, as used herein, refers to a non-wild type pattern of gene expression. It includes: expression at non-wild type levels, i.e., over or under expression; a pattern of expression that differs from wild type in terms of the time or stage at which the gene is expressed, e.g., increased or decreased expression (as compared with wild type) at a predetermined developmental period or stage; a pattern of expression. . . from wild type in terms of the effect of an environmental stimulus or extracellular stimulus on expression of the gene, e.g., a pattern of increased or decreased expression (as compared with wild type) in the presence of an increase or decrease. . .

SUMM . . . means any aspect of the expression, function, action, or regulation of the substance. The metabolism of a substance includes modifications, e.g., covalent or non-covalent modifications of the substance. The metabolism of a substance includes modifications, e.g., covalent or non-covalent modification, the substance induces in other substances. The metabolism of a substance also includes changes in the. . .

SUMM . . . DNA, and immunology, which arc within the skill of the art. Such techniques are explained fully in the literature. See e.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning; Laboratory Manual 2nd ed. (1989); DNA Cloning, Volumes I and II (D. N Glover ed. 1985); Oligonucleotide Synthesis (M. J. Gait ed, 1984); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); the series, Methods in. . . Publishing, New York.; DNA Cloning: A Practical Approach, Volumes I and II, 1985 (D. N. Glover ed.); Oligonucleotide Synthesis, 1984, (M. L. Gait ed); Transcription and Translation, 1984 (Hames and Higgins eds.); Animal Cell Culture, 1986 (R. I. Freshney ed.); Immobilized. . . Perbal, 1984, A Practical Guide to Molecular Cloning; and Gene Transfer Vectors for Mammalian Cells, 1987 (J. H. Miller and M. P. Calos eds., Cold Spring Harbor Laboratory); Any suitable materials and/or methods known to those of skill can be utilized. . .

SUMM E. faecium Genomic Sequence

SUMM This invention provides nucleotide sequences of the genome of E. faecium which thus comprises a DNA sequence library of E. faecium genomic DNA. The detailed description that follows provides nucleotide sequences of E. faecium, and also describes how the sequences were obtained and how ORFs and protein-coding sequences were identified. Also described are methods of using the disclosed E. faecium sequences in methods including diagnostic and therapeutic applications. Furthermore, the library can be used as a database for identification and comparison of medically important sequences in this and other strains of E. faecium.

SUMM To determine the genomic sequence of E. faecium, DNA was isolated from a strain of E. Faecium, Mu, and mechanically sheared by nebulization to a median size of 2 kb. Following size fractionation by gel electrophoresis,. . .

SUMM A variety of approaches are used to order the contigs so as to obtain a continuous sequence representing the entire E. faecium genome. Synthetic oligonucleotides are designed that are complementary to sequences at the end of each contig. These oligonucleotides may be hybridized to libaries of E. faecium genomic DNA in, for example, lambda phage vectors or plasmid vectors to identify clones that contain sequences corresponding to. . .

SUMM The E. faecium sequences were analyzed for the presence of open reading frames (ORFs) comprising at least 180 nucleotides. As a result. . . on stop-to-stop codon reads, it should be understood that these ORFs may not correspond to the ORF of a naturally-occurring E. faecium polypeptide. These ORFs may contain start codons which indicate the initiation of protein synthesis of a naturally-occurring E. faecium polypeptide. Such start codons within the ORFs provided herein can be identified by those of ordinary skill in the relevant art, and the resulting ORF and the encoded E. faecium polypeptide is within the

scope of this invention. For example, within the ORFs a codon such as AUG or. . . the initiation signal for protein synthesis can be identified and the portion of an ORF to corresponding to a naturally-occurring **E**. faecium polypeptide can be recognized. The predicted coding regions were defined by evaluating the coding potential of such sequences with. . .

SUMM **E**. faecium Nucleic Acids

SUMM The present invention provides a library of **E**. faecium-derived nucleic acid sequences. The libraries provide probes, primers, and markers which can be used as markers in epidemiological studies. The present invention also provides a library of **E**. faecium-derived nucleic acid sequences which comprise or encode targets for therapeutic drugs.

SUMM The nucleic acids of this invention may be obtained directly from the DNA of the above referenced **E**. faecium strain by using the polymerase chain reaction (PCR). See "PCR, A Practical Approach" (McPherson, Quirke, and Taylor, eds., IRL. . . hybridization of synthetic oligonucleotide probes to filter lifts of the library colonies or plaques as known in the art (see, **e**.g., Sambrook et al., Molecular Cloning, A Laboratory Manual 2nd edition, 1989, Cold Spring Harbor Press, N.Y.).

SUMM It is also possible to obtain nucleic acids encoding **E**. faecium polypeptides from a cDNA library in accordance with protocols herein described. A cDNA encoding an **E**. faecium polypeptide can be obtained by isolating total mRNA from an appropriate strain. Double stranded cDNAs can then be prepared from the total mRNA. Subsequently, the cDNAs can be inserted into a suitable plasmid or viral (**e**.g., bacteriophage) vector using any one of a number of known techniques. Genes encoding **E**. faecium polypeptides can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided. . .

SUMM . . . polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See **e**.g., Itakura et al. U.S. Pat. No. 4,598,049; Caruthers et al. U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796. . .

SUMM In another example, DNA can be chemically synthesized using, **e**.g., the phosphoramidite solid support method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185, the method of Yoo et. . .

SUMM . . . with the sequence of the invention contained in the Sequence Listing can be used as a probe to specifically detect **E**. faecium. With the sequence information set forth in the present application, sequences of twenty or more nucleotides are identified which provide the desired inclusivity and exclusivity with respect to **E**. faecium, and extraneous nucleic acids likely to be encountered during hybridization conditions. More preferably, the sequence will comprise at least. . .

SUMM . . . having twenty or more nucleotides in a sequence of the invention contained in the Sequence Listing have utility to separate **E**. faecium nucleic acid from the nucleic acid of each other and other organisms. Nucleic acid having twenty or more nucleotides. . .

SUMM Nucleic acid isolated or synthesized in accordance with the sequences described herein have utility as primers for the amplification of **E**. faecium nucleic acid. These nucleic acids may also have utility as primers for the amplification of nucleic acids in other. . . the invention contained in the Sequence Listing have utility in conjunction with suitable enzymes and reagents to create copies of **E**. faecium nucleic acid. More preferably, the sequence will comprise twenty or more nucleotides to convey stability to the hybridization product. . .

SUMM The copies can be used in diagnostic assays to detect specific sequences, including genes from **E**. faecium and/or other Enterococcus species. The copies can also be incorporated into cloning and expression vectors to generate polypeptides corresponding. . .

SUMM . . . isolated or synthesized in accordance with the sequences described herein have utility as antisense agents to prevent the expression of **E**. faecium genes. These sequences also have utility as antisense agents to prevent expression of genes of other Enterococcus species.

SUMM In one embodiment, nucleic acid or derivatives corresponding to **E**. faecium nucleic acids is loaded into a suitable carrier such as a liposome or bacteriophage for introduction into bacterial cells.. . .

SUMM The present invention encompasses isolated polypeptides and nucleic acids derived from **E**. faecium that are useful as reagents for diagnosis of bacterial infection, components of effective anti-bacterial vaccines, and/or as targets for anti-bacterial drugs, including anti-**E**. faecium drugs.

SUMM Expression of **E**. faecium Nucleic Acids

SUMM . . . or the Swissprot accession number (SP), the locus name (LN), Superfamily Classification (CL), the Organism (OR), Source of variant (SR), **E**.C. number (EC), the gene name (GN), the product name (PN), the Function Description (FN), the Map Position (MP), Left End. . .

SUMM . . . and sequence all the nucleic acid fragments of interest including open reading frames (ORFs) encoding a large variety proteins of **E**. faecium.

SUMM . . . SEQ ID NO: 1-SEQ ID NO: 3654and in Table 2 or fragments of said nucleic acid encoding active portions of **E**. faecium polypeptides can be cloned into suitable vectors or used to isolate nucleic acid. The isolated nucleic acid is combined. . .

SUMM . . . not limited to, Eucaryotic species such as the yeast Saccharomyces cerevisiae, Methanobacterium strains or other Archaea, and Eubacteria such as **E**. coli, B. Subtilis, S. Aureus, S. Pneumonia or Pseudomonas putida. In some cases the expression host will utilize the natural **E**. faecium promoter whereas in others, it will be necessary to drive the gene with a promoter sequence derived from the expressing organism (**e**.g., an **E**. coli beta-galactosidase promoter for expression in **E**. coli).

SUMM To express a gene product using the natural **E**. faecium promoter, a procedure such as the following can be used. A restriction fragment containing the gene of interest, together. . .

SUMM A suitable host cell for expression of a gene can be any procaryotic or eucaryotic cell. For example, an **E**. faecium polypeptide can be expressed in bacterial cells such as **E**. Coli or B. subtilis, insect cells (baculovirus), yeast, or mammalian cells such as Chinese hamster ovary cell (CHO). Other suitable. . .

SUMM . . . the pAc series (Smith et al., (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow, V. A., and Summers, **M**. D., (1989) Virology 170:31-39). Generally, COS cells (Gluzman, Y., (1981) Cell 23: 175-182) are used in conjunction with such vectors. . .

SUMM Expression in procaryotes is most often carried out in **E**. coli with either fusion or non-fusion inducible expression vectors. Fusion vectors usually add a number of NH_2 terminal amino acids. . . (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) which fuse glutathione S-transferase, maltose **E** binding protein, or protein A, respectively, to the target recombinant protein. A preferred reporter group is poly(His), which may be. . .

SUMM For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding an **E**. faecium polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be. . . antibodies specific for such polypeptides. Additionally, in many situations, polypeptides can be produced by chemical cleavage of a native protein (**e**.g., tryptic digestion) and the cleavage products can then be purified by standard techniques.

SUMM In the case of **membrane** bound proteins, these can be isolated from a host cell by contacting a **membrane**-associated protein fraction with a detergent forming a solubilized complex, where the **membrane**-associated protein is no longer entirely embedded in the **membrane** fraction and is solubilized at least to an extent which allows it to be chromatographically isolated from the **membrane** fraction. Several different criteria are used for choosing a detergent suitable for solubilizing these complexes. For example, one property considered is the ability of the detergent to solubilize the **E**. faecium protein within the **membrane** fraction at minimal denaturation of the

membrane associated protein allowing for the activity or functionality of the membrane-associated protein to return upon reconstitution of the protein. Another property considered when selecting the detergent is the critical micelle concentration. . . ease of removal after reconstitution. A third property considered when selecting a detergent is the hydrophobicity of the detergent. Typically, membrane-associated proteins are very hydrophobic and therefore detergents which are also hydrophobic, e.g., the triton series, would be useful for solubilizing the hydrophobic proteins. Another property important to a detergent can be the capability of the detergent to remove the E. faecium protein with minimal protein-protein interaction facilitating further purification. A fifth property of the detergent which should be considered is. . .

SUMM One strategy to maximize recombinant E. faecium peptide expression in E. coli is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein. . . Enzymology 185, Academic Press, San Diego, Calif. (1990) 119-128). Another strategy would be to alter the nucleic acid encoding an E. faecium peptide to be inserted into an expression vector so that the individual codons for each amino acid would be those preferentially utilized in highly expressed E. coli proteins (Wada et al., (1992) Nuc. Acids Res. 20:2111-2118). Such alteration of nucleic acids of the invention can be. . .

SUMM . . . polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See, e.g., Itakura et al. U.S. Pat. No. 4,598,049; Caruthers et al. U.S. Pat. No. 4,458,066; and Itakura U.S. Pat. Nos. 4,401,796. . .

SUMM The present invention provides a library of E. faecium-derived nucleic acid sequences. The libraries provide probes, primers, and markers which can be used as markers in epidemiological studies. The present invention also provides a library of E. faecium-derived nucleic acid sequences which comprise or encode targets for therapeutic drugs.

SUMM . . . acid sequence information provided in SEQ ID NO: 1-SEQ ID NO: 3654. For example, DNA can be chemically synthesized using, e.g., the phosphoramidite solid support method of Matteucci et al., 1981, J. Am. Chem. Soc. 103:3185, the method of Yoo et. . .

SUMM Alternatively, any site desired may be produced, e.g., by ligating nucleotide sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also be generated by the use of the polymerase chain reaction (PCR). See, e.g., Saiki et al., 1988, Science 239:48. The cleaved vector and the DNA fragments may also be modified if required by. . .

SUMM The nucleic acids of the present invention may be flanked by natural E. faecium regulatory sequences, or may be associated with heterologous sequences, including promoters, enhancers, response elements, signal sequences, polyadenylation sequences, introns,. . . or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoroamidates, carbamates, etc.) and with charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.). Nucleic acids may contain one or more additional covalently linked moieties, such as, for example, proteins (e.g., nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.), intercalators (e.g., acridine, psoralen, etc.), chelators (e.g., metals, radioactive metals, iron, oxidative metals, etc.), and alkylators. PNAs are also included. The nucleic acid may be derivatized by. . .

SUMM The invention also provides nucleic acid vectors comprising the disclosed E. faecium-derived sequences or derivatives or fragments thereof. A large number of vectors, including plasmid and fungal vectors, have been described. . .

SUMM The encoded E. faecium polypeptides may be expressed by using many known vectors, such as pUC plasmids, pET plasmids (Novagen, Inc., Madison, Wis.),. . .

SUMM . . . often include one or more replication systems for cloning or expression, one or more markers for selection in the host, **e**.g. antibiotic resistance, and one or more expression cassettes. The inserted **E**. faecium coding sequences may be synthesized by standard methods, isolated from natural sources, or prepared as hybrids, etc. Ligation of the **E**. faecium coding sequences to transcriptional regulatory elements and/or to other amino acid coding sequences may be achieved by known methods.. . .

SUMM . . . host cells include bacteria, archebacteria, fungi, especially yeast, and plant and animal cells, especially mammalian cells. Of particular interest are **E**. faecium, **E**. coli, B. Subtilis, Saccharomyces cerevisiae, Saccharomyces carlsbergensis, Schizosaccharomyces pombi, SF9 cells, C129 cells, 293 cells, Neurospora, and CHO cells, COS. . . are known in the art. Under appropriate expression conditions, host cells can be used as a source of recombinantly produced **E**. faecium-derived peptides and polypeptides.

SUMM Advantageously, vectors may also include a transcription regulatory element (i.**e**., a promoter) operably linked to the **E**. faecium portion. The promoter may optionally contain operator portions and/or ribosome binding sites. Non-limiting examples of bacterial promoters compatible with **E**. coli include: b-lactamase (penicillinase) promoter; lactose promoter; tryptophan (trp) promoter; araBAD (arabinose) operon promoter; lambda-derived P_1 promoter and N gene. . .

SUMM Nucleic acids encoding wild-type or variant **E**. faecium-derived polypeptides may also be introduced into cells by recombination events. For example, such a sequence can be introduced into. . .

SUMM The nucleic acids of the present invention find use as templates for the recombinant production of **E**. faecium-derived peptides or polypeptides.

SUMM Identification and Use of **E**. faecium Nucleic Acid Sequences

SUMM The disclosed **E**. faecium polypeptide and nucleic acid sequences, or other sequences that are contained within ORFs, including complete protein-coding sequences, of which any of the disclosed **E**. faecium-specific sequences forms a part, are useful as target components for diagnosis and/or treatment of **E**. faecium-caused infection

SUMM . . . by using an isolated nucleic acid encoding the disclosed sequence, or fragments thereof, to prime a sequencing reaction with genomic **E**. faecium DNA as template; this is followed by sequencing the amplified product. The isolated nucleic acid encoding the disclosed sequence, or fragments thereof, can also be hybridized to **E**. faecium genomic libraries to identify clones containing additional complete segments of the protein-coding sequence of which the shorter sequence forms. . .

SUMM Identification of Nucleic Acids Encoding Vaccine Components and Targets for Agents Effective Against **E**. faecium

SUMM The disclosed **E**. faecium genome sequence includes segments that direct the synthesis of ribonucleic acids and polypeptides, as well as origins of replication,. . . and intergenic nucleic acids. The invention encompasses nucleic acids encoding immunogenic components of vaccines and targets for agents effective against **E**. faecium. Identification of said immunogenic components involved in the determination of the function of the disclosed sequences, which can be. . .

SUMM Computer-assisted comparison of the disclosed **E**. faecium sequences with previously reported sequences present in publicly available databases is useful for identifying functional **E**. faecium nucleic acid and polypeptide sequences. It will be understood that protein-coding sequences, for example, may be compared as a. . . short and thus may represent only a fraction of the entire protein-coding sequence. Identification of such a feature in an **E**. faecium sequence is therefore useful in determining the function of the encoded protein and identifying useful targets of antibacterial drugs.

SUMM . . . are structural features that are common to secretory, transmembrane, and surface proteins, including secretion signal peptides and hydrophobic transmembrane domains. **E**. faecium proteins identified as containing putative signal sequences and/or transmembrane domains are useful as immunogenic components of vaccines.

SUMM . . . for therapeutic drugs according to the invention include, but

are not limited to, polypeptides of the invention, whether unique to **E**. faecium or not, that are essential for growth and/or viability of **E**. faecium under at least one growth condition. Polypeptides essential for growth and/or viability can be determined by examining the effect of deleting and/or disrupting the genes, i.**e**., by so-called gene "knockout". Alternatively, genetic footprinting can be. used (Smith et al., 1995, Proc. Natl. Acad. Sci. USA 92:5479-6433;. . . No. 5,612,180). Still other methods for assessing essentiality includes the ability to isolate conditional lethal mutations in the specific gene (**e**.g., temperature sensitive mutations). Other useful targets for therapeutic drugs, which include polypeptides that are not essential for growth or viability. . .

SUMM Because of the evolutionary relationship between different **E**. faecium strains, it is believed that the presently disclosed **E**. faecium sequences are useful for identifying, and/or discriminating between, previously known and new **E**. faecium strains. It is believed that other **E**. faecium strains will exhibit at least 70% sequence homology with the presently disclosed sequence. Systematic and routine analyses of DNA sequences derived from samples containing **E**. faecium strains, and comparison with the present sequence allows for the identification of sequences that can be used to discriminate between strains, as well as those that are common to all **E**. faecium strains. In one embodiment, the invention provides nucleic acids, including probes, and peptide and polypeptide sequences that discriminate between different strains of **E**. faecium. Strain-specific components can also be identified functionally by their ability to elicit or react with antibodies that selectively recognize one or more **E**. faecium strains.

SUMM In another embodiment, the invention provides nucleic acids, including probes, and peptide and polypeptide sequences that are common to all **E**. faecium strains but are not found in other bacterial species.

SUMM **E**. faecium Polypeptides

SUMM This invention encompasses isolated **E**. faecium polypeptides encoded by the disclosed **E**. faecium genomic sequences, including the polypeptides of the invention contained in the Sequence Listing. Polypeptides of the invention are preferably. . . using methods well-known in the art. It will be understood that the sequence of an entire nucleic acid encoding an **E**. faecium polypeptide can be isolated and identified based on an ORF that encodes only a fragment of the cognate protein-coding. . . by using the isolated nucleic acid encoding the ORF, or fragments thereof, to prime a polymerase chain reaction with genomic **E**. faecium DNA as template; this is followed by sequencing the amplified product.

SUMM The polypeptides of the present invention, including function-conservative variants of the disclosed ORFs, may be isolated from wild-type or mutant **E**. faecium cells, or from heterologous organisms or cells (including, but not limited to, bacteria, fungi, insect, plant, and mammalian cells) including **E**. faecium into which a **E**. faecium-derived protein-coding sequence has been introduced and expressed. Furthermore, the polypeptides may be part of recombinant fusion proteins.

SUMM **E**. faecium polypeptides of the invention can be chemically synthesized using commercially automated procedures such as those referenced herein , including,. . .

SUMM . . . groups are those known to be useful in the art of stepwise polypeptide synthesis. Included are acyl type protecting groups, **e**.g., formyl, trifluoroacetyl, acetyl, aromatic urethane type protecting groups, **e**.g., benzyloxycarbonyl (Cbz), substituted benzyloxycarbonyl and 9-fluorenylmethyloxycarbonyl (Fmoc), aliphatic urethane protecting groups, **e**.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl, and alkyl type protecting groups, **e**.g., benzyl, triphenylmethyl. The preferred protecting group is Boc. The side-chain protecting groups for Tyr include tetrahydropyranyl, tert-butyl, trityl, benzyl, Cbz,. . .

SUMM . . . or in DMF. CH_{2Cl2} or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage, **e**.g., by the ninhydrin reaction as described by Kaiser et al., 1970, Anal. Biochem. 34:595. In cases where incomplete coupling is. . .

SUMM . . . requires the use of an orthogonal protection scheme which enables selective cleavage of the side-chain functions of acidic amino acids (**e**.g., Asp) and the basic amino acids (**e**.g., Lys). The 9-fluorenylmethyl (Fm) protecting group for the side-chain of Asp and the 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group for the side-chain. . .

SUMM . . . and countercurrent distribution. For some purposes, it is preferable to produce the polypeptide in a recombinant system in which the **E**. faecium protein contains an additional sequence tag that facilitates purification, such as, but not limited to, a polyhistidine sequence. The. . . from a crude lysate of the host cell by chromatography on an appropriate solid-phase matrix. Alternatively, antibodies produced against a **E**. faecium protein or against peptides derived therefrom can be used as purification reagents. Other purification methods are possible.

SUMM The present invention also encompasses derivatives and homologues of **E**. faecium-encoded polypeptides. For some purposes, nucleic acid sequences encoding the peptides may be altered by substitutions, additions, or deletions that provide for functionally equivalent molecules, i.**e**., function-conservative variants. For example, one or more amino acid residues within the sequence can be substituted by another amino acid. . .

SUMM To identify **E**. faecium-derived polypeptides for use in the present invention, essentially the complete genomic sequence of a virulent, methicillin-resistant isolate of Enterococcus. . .

SUMM Also encompassed are any **E**. faecium polypeptide sequences that are contained within the open reading frames (ORFs), including complete protein-coding sequences, of which any of. . .

SUMM The present invention provides a library of **E**. faecium-derived polypeptide sequences, and a corresponding library of nucleic acid sequences encoding the polypeptides, wherein the polypeptides themselves, or polypeptides. . .

SUMM The present invention also provides a library of **E**. faecium-derived polypeptide sequences, and a corresponding library of nucleic acid sequences encoding the polypeptides, wherein the polypeptides themselves, or polypeptides. . . to any known prokaryotic or eukaryotic sequences. Such libraries provide probes, primers, and markers which can be used to diagnose **E**. faecium infection, including use as markers in epidemiological studies. Non-limiting examples of such sequences are listed by SEQ ID NO. . .

SUMM The present invention also provides a library of **E**. faecium-derived polypeptide sequences, and a corresponding library of nucleic acid sequences encoding the polypeptides, wherein the polypeptides themselves, or polypeptides. . .

SUMM The selection of candidate protein antigens for vaccine development can be derived from the nucleic acids encoding **E**. faecium polypeptides. First, the ORF's can be analyzed for homology to other known exported or **membrane** proteins and analyzed using the discriminant analysis described by Klein, et al. (Klein, P., Kanehsia, **M**., and DeLisi, C. (1985) Biochimica et Biophysica Acta.815, 468-476) for predicting exported and **membrane** proteins.

SUMM . . . a probability score which indicates the probability of finding this sequence by chance in the database. ORF's with significant homology (**e**.g. probabilities lower than 1×10^{-6} that the homology is only due to random chance) to **membrane** or exported proteins represent protein antigens for vaccine development. Possible functions can be provided to **E**. faecium genes based on sequence homology to genes cloned in other organisms.

SUMM . . . intrinsic information contained in the ORF amino acid sequence and compares it to information derived from the properties of known **membrane** and exported proteins. This comparison predicts which proteins will be exported, **membrane** associated or cytoplasmic. ORF amino acid sequences identified as exported or **membrane** associated by this algorithm are likely protein antigens for vaccine development.

SUMM Production of Fragments and Analogs of **E**. faecium Nucleic Acids and Polypeptides

SUMM Based on the discovery of the **E.** faecium gene products of the invention provided in the Sequence Listing, one skilled in the art can alter the disclosed structure (of **E.** faecium genes), **e.g.**, by producing fragments or analogs, and test the newly produced structures for activity. Examples of techniques known to those skilled. . . fragments and analogs are discussed below. These, or analogous methods can be used to make and screen libraries of polypeptides, **e.g.**, libraries of random peptides or libraries of fragments or analogs of cellular proteins for the ability to bind **E.** faecium polypeptides. Such screens are useful for the identification of inhibitors of **E.** faecium.

SUMM Fragments of a protein can be produced in several ways, **e.g.**, recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing. . .

SUMM . . . amplified using the polymerase chain reaction (PCR) under conditions that reduce the fidelity of DNA synthesis by Taq DNA polymerase, **e.g.**, by using a dGTP/dATP ratio of five and adding Mn^{2+} to the PCR reaction. The pool of amplified DNA fragments. . .

SUMM . . . of single base substitutions into cloned DNA fragments (Mayers et al., 1985, Science 229:242). This technique includes generation of mutations, **e.g.**, by chemical treatment or irradiation of single-stranded DNA in vitro, and synthesis of a complimentary DNA strand. The mutation frequency. . .

SUMM . . . used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, **e.g.**, deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, **e.g.**, by (1) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (2) deleting. . .

SUMM . . . for mutagenesis, Cunningham and Wells (Science 244:1081-1085, 1989). In alanine scanning, a residue or group of target residues are identified (**e.g.**, charged residues such as Arg, Asp, His, Lys, and Glu) and replaced by a neutral or negatively charged amino acid. . .

SUMM Oligonucleotide-mediated mutagenesis is a useful method for preparing substitution, deletion, and insertion variants of DNA, see, **e.g.**, Adelman et al., (DNA 2:183, 1983). Briefly, the desired DNA is altered by hybridizing an oligonucleotide encoding a mutation to. . .

SUMM Other Modifications of **E.** faecium Nucleic Acids and Polypeptides

SUMM It is possible to modify the structure of an **E.** faecium polypeptide for such purposes as increasing solubility, enhancing stability (**e.g.**, shelf life ex vivo and resistance to proteolytic degradation in vivo). A modified **E.** faecium protein or peptide can be produced in which the amino acid sequence has been altered, such as by amino. . .

SUMM An **E.** faecium peptide can also be modified by substitution of cysteine residues preferably with alanine, serine, threoine, leucine or glutamic acid. . .

SUMM In order to enhance stability and/or reactivity, an **E.** faecium polypeptide can be modified to incorporate one or more polymorphisms in the amino acid sequence of the protein resulting. . . acid analogs can be substituted or added to produce a modified protein within the scope of this invention. Furthermore, an **E.** faecium polypeptide can be modified using polyethylene glycol (PEG) according to the method of A. Sehon and co-workers (Wie et. . . a protein conjugated with PEG. In addition, PEG can be added during chemical synthesis of the protein. Other modifications of **E.** faecium proteins include reduction/alkylation (Tarr, Methods of Protein Microcharacterization, J. **E.** Silver ed., Humana Press, Clifton N.J. 155-194 (1986)); acylation (Tarr, supra); chemical coupling to an appropriate carrier (Mishell and Shiigi,. . .

SUMM To facilitate purification and potentially increase solubility of an **E.** faecium protein or peptide, it is possible to add an amino acid fusion moiety to the peptide backbone. For example, hexa-histidine can be added to the protein for purification by immobilized metal ion

affinity chromatography (Hochuli, E. et al., (1988) Bio/Technology, 6: 1321-1325). In addition, to facilitate isolation of peptides free of irrelevant sequences, specific endoprotease cleavage. . .

SUMM To potentially aid proper antigen processing of epitopes within an **E**. faecium polypeptide, canonical protease sensitive sites can be engineered between regions, each comprising at least one epitope via recombinant or. . .

SUMM . . . cells with the resulting library of vectors, and expressing the genes under conditions in which detection of a desired activity, **e**.g., in this case, binding to **E**. faecium polypeptide or an interacting protein, facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the techniques described below is amenable to high through-put analysis for screening large numbers of sequences created, **e**.g., by random mutagenesis techniques.

SUMM . . . such as the system described above (as with the other screening methods described herein), can be used to identify polypeptides, **e**.g., fragments or analogs of a naturally-occurring **E**. faecium polypeptide, **e**.g., of cellular proteins, or of randomly generated polypeptides which bind to an **E**. faecium protein. (The **E**. faecium domain is used as the bait protein and the library of variants are expressed as prey fusion proteins.) In. . . two hybrid assay (as with the other screening methods described herein), can be used to find polypeptides which bind a **E**. faecium polypeptide.

SUMM . . . is detected in a "panning assay". For example, the gene library can be cloned into the gene for a surface **membrane** protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et. . . a similar fashion, a detectably labeled ligand can be used to score for potentially functional peptide homologs. Fluorescently labeled ligands, **e**.g., receptors, can be used to detect homologs which retain ligand-binding activity. The use of fluorescently labeled ligands, allows cells to. . .

SUMM . . . affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical **E**. coli filamentous phages M13, fd., and f1 are most often used in phage display libraries. Either of the phage gIII. . .

SUMM A common approach uses the maltose receptor of **E**. coli (the outer **membrane** protein, LamB) as a peptide fusion partner (Charbit et al. (1986) EMBO 5, 3029-3037). Oligonucleotides have been inserted into plasmids. . . produce peptides fused into one of the extracellular loops of the protein. These peptides are available for binding to ligands, **e**.g., to antibodies, and can elicit an immune response when the cells are administered to animals. Other cell surface proteins, **e**.g., OmpA (Schorr et al. (1991) Vaccines 91, pp. 387-392), PhoE (Agterberg, et al. (1990) Gene 88, 37-45), and PAL (Fuchs. . . of other bacterial species have also served as peptide fusion partners. Examples include the Staphylococcus protein A and the outer **membrane** IgA protease of Neisseria (Hansson et al. (1992) J. Bacteriol. 174, 4239-4245 and Klauser et al. (1990) EMBO J. 9,. . .

SUMM . . . cells. The phage coat fusions are exposed briefly to the cytoplasm during translation but are rapidly secreted through the inner **membrane** into the periplasmic compartment, remaining anchored in the **membrane** by their C-terminal hydrophobic domains, with the N-termini, containing the peptides, protruding into the periplasm while awaiting assembly into phage. . . significantly as a result of their exposure to different proteolytic activities. The phage coat proteins require transport across the inner **membrane** and signal peptidase processing as a prelude to incorporation into phage. Certain peptides exert a deleterious effect on these processes. . .

SUMM . . . (1994) J. Med. Chem. 37(9):1233-1251), a molecular DNA library encoding 10^{12} decapeptides was constructed and the library expressed in an **E**. coli S30 in vitro coupled transcription/translation system. Conditions were chosen to stall the ribosomes on the mRNA, causing the accumulation. . .

SUMM . . . high through-put assays described above can be followed by secondary screens in order to identify further biological activities

which will, e.g., allow one skilled in the art to differentiate agonists from antagonists. The type of a secondary screen used will depend. . .

SUMM Peptide Mimetics of **E**. faecium Polypeptides

SUMM The invention also provides for reduction of the protein binding domains of the subject **E**. faecium polypeptides to generate mimetics, **e**.g. peptide or non-peptide agents. The peptide mimetics are able to disrupt binding of a polypeptide to its counter ligand, **e**.g., in the case of an **E**. faecium polypeptide binding to a naturally occurring ligand. The critical residues of a subject **E**. faecium polypeptide which are involved in molecular recognition of a polypeptide can be determined and used to generate **E**. faecium-derived peptidomimetics which competitively or noncompetitively inhibit binding of the **E**. faecium polypeptide with an interacting polypeptide (see, for example, European patent applications EP-412,762A and EP-B31,080A).

SUMM For example, scanning mutagenesis can be used to map the amino acid residues of a particular **E**. faecium polypeptide involved in binding an interacting polypeptide, peptidomimetic compounds (**e**.g. diazepine or isoquinoline derivatives) can be generated which mimic those residues in binding to an interacting polypeptide, and which therefore can inhibit binding of an **E**. faecium polypeptide to an interacting polypeptide and thereby interfere with the function of **E**. faecium polypeptide. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (**e**.g., see Freidinger et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (**e**.g., see Huffman et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama. . .

SUMM Vaccine Formulations for **E**. faecium Nucleic Acids and Polypeptides

SUMM This invention also features vaccine compositions for protection against infection by **E**. faecium or for treatment of **E**. faecium infection, a gram-negative spiral microaerophilic bacterium. In one embodiment, the vaccine compositions contain one or more immunogenic components such as a surface protein from **E**. faecium, or portion thereof, and a pharmaceutically acceptable carrier. Nucleic acids within the scope of the invention are exemplified by the nucleic acids of the invention contained in the Sequence Listing which encode **E**. faecium surface proteins. Any nucleic acid encoding an immunogenic **E**. faecium protein, or portion thereof, which is capable of expression in a cell, can be used in the present invention.. . .

SUMM One aspect of the invention provides a vaccine composition for protection against infection by **E**. faecium which contains at least one immunogenic fragment of an **E**. faecium protein and a pharmaceutically acceptable carrier. Preferred fragments include peptides of at least about 10 amino acid residues in. . .

SUMM . . . be obtained, for example, by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding the full-length **E**. faecium protein. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid. . .

SUMM . . . or smallest unit of recognition by a T cell receptor, where the epitope comprises amino acids essential to receptor recognition (**e**.g., approximately 6 or 7 amino acid residues). Amino acid sequences which mimic those of the T cell epitopes are within. . .

SUMM . . . immunogenic with an antigen presenting cell which presents appropriate MHC molecules in a T cell culture. Presentation of an immunogenic **E**. faecium peptide in association with appropriate MHC molecules to T cells in conjunction with the necessary co-stimulation has the effect. . .

SUMM Vaccine compositions of the invention containing immunogenic components (**e**.g., **E**. faecium polypeptide or fragment thereof or nucleic acid encoding an **E**. faecium polypeptide or fragment thereof) preferably include a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable carrier" refers to a carrier. . . agents, preservatives or buffers, which enhance the shelf life or effectiveness of the antibody. For vaccines of the invention containing

E. faecium polypeptides, the polypeptide is co-administered with a suitable adjuvant.

SUMM Vaccine compositions are conventionally administered parenterally, e.g., by injection, either subcutaneously or intramuscularly. Methods for intramuscular immunization are described by Wolff et al. (1990) Science 247: 1465-1468. . . and pulmonary formulations, suppositories, and transdermal applications. Oral immunization is preferred over parenteral methods for inducing protection against infection by E. faecium Cain et. al. (1993) Vaccine 11: 637-642. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades. . .

SUMM . . . be used are non-toxic derivatives of cholera toxin, including its B subunit, and/or conjugates or genetically engineered fusions of the E. faecium polypeptide with cholera toxin or its B subunit, procholeragenoid, fungal polysaccharides, including schizophyllan, muramyl dipeptide, muramyl dipeptide derivatives, phorbol esters, labile toxin of E. coli, non-E. faecium bacterial lysates, block polymers or saponins.

SUMM . . . complexes (ISCOMs), cochleates, or liposomes, genetically engineered attenuated live vectors such as viruses or bacteria, and recombinant (chimeric) virus-like particles, e.g., bluetongue. The amount of adjuvant employed will depend on the type of adjuvant used. For example, when the mucosal adjuvant. . .

SUMM . . . systems in humans may include enteric release capsules protecting the antigen from the acidic environment of the stomach, and including E. faecium polypeptide in an insoluble form as fusion proteins. Suitable carriers for the vaccines of the invention are enteric coated. . .

SUMM . . . be administered as a primary prophylactic agent in adults or in children, as a secondary prevention, after successful eradication of E. faecium in an infected host, or as a therapeutic agent in the aim to induce an immune response in a susceptible host to prevent infection by E. faecium. The vaccines of the invention are administered in amounts readily determined by persons of ordinary skill in the art.. . . levels can be obtained based on results with known oral vaccines such as, for example, a vaccine based on an E. coli lysate (6 mg dose daily up to total of 540 mg) and with an enterotoxigenic E. coli purified antigen (4 doses of 1 mg) (Schulman et al., J. Urol. 150:917-921 (1993); Boedecker et al., American Gastroenterological. . .

SUMM In a preferred embodiment, a vaccine composition of the invention can be based on a killed whole E. coli preparation with an immunogenic fragment of an E. faecium protein of the invention expressed on its surface or it can be based on an E. coli lysate, wherein the killed E. coli acts as a carrier or an adjuvant.

SUMM . . . to those skilled in the art that some of the vaccine compositions of the invention are useful only for preventing E. faecium infection, some are useful only for treating E. faecium infection, and some are useful for both preventing and treating E. faecium infection. In a preferred embodiment, the vaccine composition of the invention provides protection against E. faecium infection by stimulating humoral and/or cell-mediated immunity against E. faecium. It should be understood that amelioration of any of the symptoms of E. faecium infection is a desirable clinical goal, including a lessening of the dosage of medication used to treat E. faecium-caused disease, or an increase in the production of antibodies in the serum or mucous of patients.

SUMM Antibodies Reactive With E. faecium Polypeptides

SUMM The invention also includes antibodies specifically reactive with the subject E. faecium polypeptide. Anti-protein/anti-peptide antisera or monoclonal antibodies can be made by standard protocols (See, for example, Antibodies: A Laboratory Manual. . . or peptide include conjugation to carriers or other techniques well known in the art. An immunogenic portion of the subject E. faecium polypeptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of. . .

SUMM In a preferred embodiment, the subject antibodies are immunospecific for

antigenic determinants of the E. faecium polypeptides of the invention, e.g. antigenic determinants of a polypeptide of the invention contained in the Sequence Listing, or a closely related human or non-human mammalian homolog (e.g., 90% homologous, more preferably at least 95% homologous). In yet a further preferred embodiment of the invention, the anti-E. faecium antibodies do not substantially cross react (i.e., react specifically) with a protein which is for example, less than 80% percent homologous to a sequence of the invention. . .

SUMM The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with E. faecium polypeptides. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as. . . to produce Fab' fragments. The antibody of the invention is further intended to include bispecific and chimeric molecules having an anti-E. faecium portion.

SUMM Both monoclonal and polyclonal antibodies (Ab) directed against E. faecium polypeptides or E. faecium polypeptide variants, and antibody fragments such as Fab' and $F(ab')_2$, can be used to block the action of E. faecium polypeptide and allow the study of the role of a particular E. faecium polypeptide of the invention in aberrant or unwanted intracellular signaling, as well as the normal cellular function of the E. faecium and by microinjection of anti-E. faecium polypeptide antibodies of the present invention.

SUMM Antibodies which specifically bind E. faecium epitopes can also be used in immunohistochemical staining of tissue samples in order to evaluate the abundance and pattern of expression of E. faecium antigens. Anti E. faecium polypeptide antibodies can be used diagnostically in immuno-precipitation and immuno-blotting to detect and evaluate E. faecium levels in tissue or bodily fluid as part of a clinical testing procedure. Likewise, the ability to monitor E. faecium polypeptide levels in an individual can allow determination of the efficacy of a given treatment regimen for an individual afflicted with such a disorder. The level of an E. faecium polypeptide can be measured in cells found in bodily fluid, such as in urine samples or can be measured in tissue, such as produced by gastric biopsy. Diagnostic assays using anti-E. faecium antibodies can include, for example, immunoassays designed to aid in early diagnosis of E. faecium infections. The present invention can also be used as a method of detecting antibodies contained in samples from individuals infected by this bacterium using specific E. faecium antigens.

SUMM Another application of anti-E. faecium polypeptide antibodies of the invention is in the immunological screening of cDNA libraries constructed in expression vectors such as. . . consist of β-galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of a subject E. faecium polypeptide can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with anti-E. faecium polypeptide antibodies. Phage, scored by this assay, can then be isolated from the infected plate. Thus, the presence of E. faecium gene homologs can be detected and cloned from other species, and alternate isoforms (including splicing variants) can be detected. . .

SUMM Drug Screening Assays Using E. faecium Polypeptides

SUMM By making available purified and recombinant E. faecium polypeptides, the present invention provides assays which can be used to screen for drugs which are either agonists or antagonists of the normal cellular function, in this case, of the subject E. faecium polypeptides, or of their role in intracellular signaling. Such inhibitors or potentiators may be useful as new therapeutic agents to combat E. faecium infections in humans. A variety of assay formats will suffice and, in light of the present inventions, will be. . .

SUMM . . . in an exemplary screening assay of the present invention, the compound of interest is contacted with an isolated and purified E. faecium polypeptide.

SUMM Screening assays can be constructed in vitro with a purified E. faecium polypeptide or fragment thereof, such as an E. faecium

polypeptide having enzymatic activity, such that the activity of the polypeptide produces a detectable reaction product. The efficacy of. . . naturally occurring compounds can be tested in the assay to identify those which inhibit or potentiate the activity of the **E**. faecium polypeptide. Some of these active compounds may directly, or with chemical alterations to promote **membrane** permeability or solubility, also inhibit or potentiate the same activity (**e**.g., enzymatic activity) in whole, live **E**. faecium cells.

SUMM For general information concerning formulations, see, **e**.g., Gilman et al. (eds.), 1990, Goodman and Gilman's: The Pharmacological Basis of Therapeutics, 8th ed., Pergamon Press; and Remington's Pharmaceutical. . .

SUMM The antibacterial agents and compositions of the present invention are useful for preventing or treating **E**. faecium infections. Infection prevention methods incorporate a prophylactically effective amount of an antibacterial agent or composition. A prophylactically effective amount is an amount effective to prevent **E**. faecium infection and will depend upon the specific bacterial strain, the agent, and the host. These amounts can be determined. . .

SUMM **E**. faecium infection treatment methods incorporate a therapeutically effective amount of an antibacterial agent or composition. A therapeutically effective amount is. . .

DETD I. Cloning and Sequencing of **E**. faecium DNA

DETD **E**. faecium chromosomal DNA was isolated according to a basic DNA protocol outlined in Schleif R. F. and Wensink P. C.,. . . Briefly, cells were pelleted, resuspended in TE (10 mM Tris, 1 mM EDTA, pH 7.6) and GES lysis buffer (5.1 **M** guanidium thiocyanate, 0.1 **M** EDTA, pH 8.0, 0.5% N-laurylsarcosine) was added. Suspension was chilled and ammonium acetate (NH4Ac) was added to final concentration of 2.0 **M**. DNA was extracted, first with chloroform, then with phenol-chloroform, and reextracted with chloroform. DNA was precipitated with isopropanol, washed twice. . .

DETD Following isolation whole genomic **E**. faecium DNA was nebulized (Bodenteich et al., Automated DNA Sequencing and Analysis (J. C. Venter, ed.), Academic Press, 1994) to. . .

DETD All subsequent steps were based either on the multiplex DNA sequencing protocols outlined in Church G. **M**. and Kieffer-Higgins S., Science 240:185-188, 1988 or by ABI377 automated DNA sequencing methods. Only major modifications to the protocols are. . .

DETD The purified DNA samples were then sequenced either using the multiplex DNA sequencing based on chemical degradation methods (Church G. **M**. and Kieffer-Higgins S., Science 240:185-188, 1988) or by Sequithrem (Epicenter Technologies) dideoxy sequencing protocols or by ABI dye-terminator chemistry. For. . . were electrophoresed and transferred onto nylon membranes by direct transfer electrophoresis from 40 cm^2 gels (Richterich P. and Church G. **M**., Methods in Enzymology 218:187-222, 1993). The DNA was covalently bound to the membranes by exposure to ultraviolet light, and hybridized. . . hybridized probe was removed by incubation at 65° C., and the hybridization cycle repeated with another tag sequence until the **membrane** had been probed 41 times. Thus, each gel produced a large number of films, each containing new sequencing information. Whenever. . .

DETD To identify **E**. faecium polypeptides the complete genomic sequence of **E**. faecium were analyzed essentially as follows: First, all possible stop-to- stop open reading frames (ORFs) greater than 180 nucleotides in. . .

DETD Identification, Cloning and Expression of **E**. faecium Nucleic Acids

DETD Expression and purification of the **E**. faecium polypeptides of the invention can be performed essentially as outlined below.

DETD To facilitate the cloning, expression and purification of **membrane** and secreted proteins from **E**. faecium, a gene expression system, such as the pET System (Novagen), for cloning and expression of recombinant proteins in **E**. coli, is selected. Also, a DNA sequence encoding a peptide tag, the His-Tag, is fused to the 3' end of. . . the recombinant protein products. The 3' end is selected for fusion in order

to avoid alteration of any 5' terminal signal sequence.

DETD . . . the nucleic acids set forth in SEQ ID NO: 1-SEQ ID NO: 3654) for cloning from the Mu strain of **E**. faecium are prepared for amplification cloning by polymerase chain reaction (PCR). Synthetic oligonucleotide primers specific for the 5' and 3'. . . translation at a methionine residue followed by a valine residue and the coding sequence for the remainder of the native **E**. faecium DNA sequence. All reverse primers (specific for the 3' end of any **E**. faecium ORF) include a EcoRI site at the extreme 5' terminus to permit cloning of each **E**. faecium sequence into the reading frame of the pET-28b. The pET-28b vector provides sequence encoding an additional 20 carboxy-terminal amino. . .

DETD Genomic DNA prepared from the Mu strain of **E**. faecium is used as the source of template DNA for PCR amplification reactions (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., F. Ausubel et al.; eds., 1994). To amplify a DNA sequence containing an **E**. faecium ORF, genomic DNA (50 nanograms) is introduced into a reaction vial containing 2 mM $MgCl_2$, 1 micromolar synthetic oligonucleotide primers (forward and reverse primers) complementary to and flanking a defined **E**. faecium ORF, 0.2 mM of each deoxynucleotide triphosphate; dATP, dGTP, dCTP, dTTP and 2.5 units of heat stable DNA polymerase. . .

DETD . . . Spin PCR purification kit (Qiagen, Gaithersburg, Md., USA). All amplified DNA samples are subjected to digestion with the restriction endonucleases, **e**.g., NcoI and EcoRI (New England BioLabs, Beverly, Mass., USA)(Current Protocols in Molecular Biology, John Wiley and Sons, Inc., F. Ausubel. . .

DETD Cloning of **E**. faecium Nucleic Acids Into an Expression Vector

DETD The pET-28b vector is prepared for cloning by digestion with endonucleases, **e**.g., NcoI and EcoRI (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., F. Ausubel et al., eds., 1994). The. . .

DETD . . . the previously digested pET-28b expression vector. Products of the ligation reaction are then used to transform the BL21 strain of **E**. coli (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., F. Ausubel et al., eds., 1994) as described below.

DETD Competent bacteria, **E** coli strain BL21 or **E**. coli strain BL21(DE3), are transformed with recombinant pET expression plasmids carrying the cloned **E**. faecium sequences according to standard methods (Current Protocols in Molecular, John Wiley and Sons, Inc., F. Ausubel et al., eds.,. . .

DETD Identification Of Recombinant Expression Vectors With **E**. faecium Nucleic Acids

DETD Individual BL21 clones transformed with recombinant pET-28b **E**. faecium ORFs are analyzed by PCR amplification of the cloned inserts using the same forward and reverse primers, specific for each **E**. faecium sequence, that were used in the original PCR amplification cloning reactions. Successful amplification verifies the integration of the **E**. faecium sequences in the expression vector (Current Protocols in Molecular Biology, John Wiley and Sons, Inc., F. Ausubel et al.,. . .

DETD Individual clones of recombinant pET-28b vectors carrying properly cloned **E**. faecium ORFs are picked and incubated in 5 mls of LB broth plus 25 microgram/ml kanamycin sulfate overnight. The following. . .

DETD Expression Of Recombinant **E**. faecium Sequences in **E**. coli

DETD The pET vector can be propagated in any **E**. coli K-12 strain **e**.g. HMS174, HB101, JM109, DH5, etc. for the purpose of cloning or plasmid preparation. Hosts for expression include **E**. coli strains containing a chromosomal copy of the gene for T7 RNA polymerase. These hosts are lysogens of bacteriophage DE3,. . .

DETD To express recombinant **E**. faecium sequences, 50 nanograms of plasmid DNA isolated as described above is used to transform competent BL21(DE3) bacteria as described. . . part of the pET expression system kit). The lacZ gene (beta-galactosidase) is expressed in the pET-System as described for the **E**. faecium recombinant constructions. Transformed cells are cultured in SOC medium for 1 hour, and the culture is then plated on. . . at which point, 1 millimolar IPTG was added to the

culture for 3 hours to induce gene expression of the E. faecium recombinant DNA constructions.

DETD . . . for 15 minutes at 4° C. Pellets are resuspended in 50 milliliters of cold 10 mM Tris-HCl, pH 8.0, 0.1 M NaCl and 0.1 mM EDTA (STE buffer). Cells are then centrifuged at 2000×g for 20 min at 4° C. Wet. . .

DETD . . . art can be utilized to purify the isolated proteins. (Current Protocols in Protein Science, John Wiley and Sons, Inc., J. E. Coligan et al., eds., 1995). For example, the frozen cells may be thawed, resupended in buffer and ruptured by several passages through a small volume microfluidizer (Model M-110S, Microfluidics International Corporation, Newton, Mass.). The resultant homogenate may be centrifuged to yield a clear supernatant (crude extract) and following. . .

DETD . . . content (Perkins, S. J. 1986 Eur. J. Biochem. 157, 169-180). Protein concentrations are also measured by the method of Bradford, M. M. (1976) Anal. Biochem. 72, 248-254, and Lowry, O. H., Rosebrough, N., Farr, A. L. & Randall, R. J. (1951) J.. . .

DETD . . . BioRad (Hercules, Calif., USA), and stained with Coomassie blue. Molecular weight markers may include rabbit skeletal muscle myosin (200 kDa), E. coli (-galactosidase (116 kDa), rabbit muscle phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), bovine carbonic. . .

DETD . . . 8.20E-06 [ac:f69921][pn:conserved hypothetical protein yoqw][gn:yoqw]
[or:bacillus subtilis][db:pir]

1054713_c1_11 41 3695 258 85 62 0.29 [ln:msgtcwpa][ac:m15467][pn:unknown protein][or:mycobacterium
tuberculosis][sr:mycohacterium tuberculosis (strain erdman) dna]
[db:genpept-bct][de:m.tuberculosis 65 kda antigen (cell wall protein a) gene.][nt:orf d158; putative][le:1194][re:1670]

1054827_f2_9 42 3696 681 226 305 2.80E-27 [ac:h69830][pn:conserved hypothetical protein yhfk][gn:yhfk]
[or:bacillus subtilis][db:pir]

10548902_f2_5 43 3697 720 239 74 0.034 [ln:lbphig1e][ac:x98106][gn:rorf58][or:bacteriophage phig 1 e]
[db:genpept-phg][de:lactobacillus bacteriophage phig 1 e complete genomic dna.][le:42253:1][re:42259:170][di:complementjoin]

10553593_f2_30 44 3698 300 99 157 1.30E-11 [ac:s76074][pn:hypothetical protein][or:syncchocystis sp.][sr:pcc
6803, , pcc 6803][sr:pcc 6803,][db:pir]

1055442_c1_86 45 3699 525. . . 83 3737 756 251 508 8.60E-49
[ac:g70066][pn:capsular polysaccharide biosynthesis homolog ywqd][gn:ywqd][or:bacillus subtilis][db:pir]

1071067_c2_104 84 3738 684 227 317 1.50E-28 [ac:p08188][gn:manz:ptsm:gptb][or:escherichia coli][de:
(eii-m-man)][sp:p08188][db:swissprot]

10718752_c3_17 85 3739 474 158 393 1.30E-36 [ln:af030359][ac:af030359][pn:dtdp-1-rhamnose synthase][gn:cpso]
[or:streptococcus pneumoniae][db:genpept-bct][de:streptococcus pneumoniae strain nctc11906 glucose-1-phosphatethymidyl transferase (cpsl) gene, partial cds; anddtdp-4-keto-6-deoxyglucos

10719675_c3_129. . .

DETD [ac:a69661][pn:transcriptional regulator msmr][gn:msmr][or:
bacillus subtilis][db:pir]

10938292_f1_2 116 3770 345 114 72 0.3 [ln:mtrcp][ac:x63508][or:mycobacterium tuberculosis][db:
genpept-bct][de:m.tuberculosis gc rich repetitive dna.][nt:predicted orf][le:251][re:703][di:direct]

10940688_c2_223 117 3771 780 259 148 4.50E-09 [ln:lpatovgns][ac:x94434][pn:plnu][gn:plnu][fn:unknown][or:
lactobacillus plantarum][db:genpept-bct][de:l.plantarum
pln[a,b,c,d,e,f,g,h,i,j,k,i,m,n,o,p,r,s,t,u,v]genesand orf1.]
[nt:putative][le:15253][re:15921][di:direct]

10948376_f1_5 118 3772 324 107 53 0.78 [in:hssphar][ac:x82554][gn:sphar][or:homo sapiens][sr:human][db:
genpept-pri2][de:h.sapiens sphar gene for cyclin-related protein.]

[sp:q15515][le:1164][re:1555][di:direct]
10972158_f1_1 119 3773 1050. . . protein ydao][gn:ydao][or:
bacillus subtilis][db:pir]
114813_f2_32 140 3794 540 179 353 2.30E-32 [ac:f69744][pn:hypothetical protein
ybbk][gn:ybbk][or:bacillus
subtilis][db:pir]
171961_f3_45 141 3795 186 61 61 0.47 [ln:ecotolqra][ac:m
16489][or:escherichia coli][sr:escherichia coli
dna][db:genpept-bct][de:escherichia coli tolqra gene cluster dna.]
[nt:orf 4; putative][le:627][re:1199][di:complement]
1720053_c3_22 142 3796 1227 408 1664 2.70E-171 [ac:p26235][gn:napa][or:enteroco
ccus hirae][de:na(+)/h(+)
. . . 0.28 [ln:hsenac07][ac:u53841][pn:amiloride-sensitive epithelial sodium
channel][gn:scnn1g][or:homo sapiens][sr:human][db:genpept-
pri2l [de:human amiloride-sensitive epithelial sodium channel
gamma subunit(scnn1g) gene, partial exon 4 and intron 4.][nt:e
1175293_c1_50 147 3801 621 206 412 1.30E-38 [ln:sadired][ac:z16422][pn:unknown]
[gn:orf2][or:staphylococcus
aureus][db:genpept-bct][de:s.aureus dfrb gene for dihydrofolate
reductase.][le:646][re:1230][di:direct]
11758465_c3_55 148 3802 225 74 57 0.017 [ln:ae001124][ac:ac001124:ae000783][pn:
conserved. . . precursor]
[cl:circumsporozoite protein:thrombospondin type 1 repeat
homology][or:plasmodium vivax][db:pir]
11850892_c3_30 168 3822 219 72 63 0.13 [ln:efpad 1 or f][ac:x96977][gn:orf1
1][or:enterococcus faccalis]
[db:genpept-bct][de:e.faecalis plasmid pad1, open reading frames.]
[le:4168][re:4539][di:direct]
11879036_f1_2 169 3823 213 70 59 0.6 [ln:chkestpcsc][ac:d26313[[pn:unknown
protein; incomplete]
[or:gallus gallus][sr:gallus gallus lens fibers. . . site)
lyase)][sp:p39788][db:swissprot]
1204777_ci _6 190 3844 303 100 89 0.003 [ln:chu45963][ac:u45963][pn:lmp1][gn:lm
p1][or:cercopithecine
herpesvirus 15][sr:rhesus epstein barr virus strain=lc18664]
[db:genpept-vrl][de:cercopithecine herpesvirus 15 latent membrane
protein 1 homolog(lmp1) gene, complete cds.][nt:latent membr
1205302_c2_91 191 3845 459 152 470 9.10E-45 [ac:p42980][gn:mgsa][or:bacillus
subtilis][cc:4.2.99.11]
[de:methylglyoxal synthase,][sp:p42980][db:swissprot]
1205311_c2_36 192 3846 1557. . . dna encoding the arginine-deiminase
pathwaygenes.][le:1503][re:2516][di:direct]
12116083_f2_18 199 3853 471 156 226 6.60E-19 [ln:cfu09422][ac:u09422][or:entero
coccus faecalis][db:genpept bet]
[de:enterococcus faecalis ds 16 transposon tn916, (tet(m)), (xis-tn),
(int-tn) genes, orfs 1-24, complete cds, complete sequence.][nt:
orf7][le:151951][re:15668][di:direct]
1213957_c1_55 200 3854 441 146 105 4.40E-06 [ln:lbphig1e][ac:x98106][gn:rorf115
][or:bacteriophage phig1e]
[db:genpept-phg][de:lactobacillus bacteriophage. . .
protein][or:syncchocystis sp.][sr:pcc
6803, , pcc 6803][sr:pcc 6803,][db:pir]
12275257_c3_200 208 3862 2451 816 3343 0 [ln:cfu09422][ac:u09422][or:enterococc
us faccalis][db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tct(m)),(xis-tn),
(int-tn) genes, orfs 1-24, comptete cds, complete sequence.]
[nt:orf16][le:5193][re:7640][di:direct]
12284377_c1 _56 209 3863 183 60 67 0.045 [ln:synd2sv1][ac:m10978][or:artificial
sequence][sr:adenovirus
type 2/simian. . . 381 1319 9.90E-135 [ac:p45872][gn:prfa][or:bacillu
s subtilis][de:peptide chain release
factor 1 (rf-1)][sp:p45872][db:swissprot]
12509702_c1_48 224 3878 1206 401 1141 7.20E-116 [ac:p35855][gn:dltb][or:tactoba
cillus casci][de:dltb protein (basic
membrane protein) (bmp)][sp:p35855][db:swissprot]
125200_c1_24 225 3879 285 94 240 2.20E-20 [ac:s42932][pn:probable transmembrane

protein smpb][gn:smpb]
[or:staphylococcus hominis][db:pir]
12526452_f2_58 226 3880 825 274 131 8.90E-06 [ln:cazp2][ac:z72495][pn:zp2][gn:z
p2][fn:egg **membrane** protein]
[or:carassius auratus][sr:goldfish][db:genpept-vrt][de:c.auratus
mrna for zp2.][le:<1][re: 1744][di:direct]
12535713_13_13 227 3881 990 329 342 2.50E-43 [ln:aty14325][ac:y14325][pn:mevalo
nate diphosphate
decarboxylase][gn:mvd1][or:arabidopsis thaliana][sr:thale
DETD . . . [ln:pyu36927][ac:u36927][pn:rhoptry protein][fn:erythrocyte
invasion and possible binding][or:plasmodiumi yoelii][db:
genpept-inv][de:plasmodium yoelii rhoptry protein gene, partial
cds.][le:<1][re:7206][di:direct]
1370317_c2_71 306 3960 537 178 139 3.30E-09 [ac:f69456][pn:**signal sequence**
peptidase homolog][or:
archaeoglobus fulgidus][db:pir]
13703383_f2_27 307 3961 1566 521 927 3.40E-93 [ac:p54718][gn:yfib][or:bacillus
subtilis][de:hypothetical abc
transporter atp-binding protein 1 in glvbe 3'region][sp:p54718]
. . .
DETD . . . 61 59 0.52 [ac:q45479][gn:lspa:lsp][or:bacillus
subtilis][ec:3.4.23.36][de:
peptidase) (signal peptidase ii) (spase ii)][sp:q45479][db:swissprot]
14109686_f1_15 383 4037 236 79 60 0.047 [ln:mbhrded][ac:y09870][pn:heterodisulf
ide reductase][gn:hdre]
[or:methanosarcina harkeri][db:genpept-bct][dc:**m**.barkeri hdre &
hdrd genes, orf1, orf2, orf3 & orf4.][le:1658][re:2449][di:direct]
14110641 _c1_202 384 4038 288 95 71 0.24 [ac:p07379][gn:pckl][or:rattus
norvegicus][sr:,rat][ec:4.1.1.32][de:
(phosphocnolpyruvate carboxylase). . . dna][db:genpept-bct][de:
enterococcus faecalis plasmid pad 1 gene.][nt:structural gene for
ultraviolet resistance][le:1284][re:2612][di:direct]
1414077_c1_176 387 4041 1002 333 138 5.60E-07 [ln:ecoadkvis][ac:d90259][pn:lipa
se like enzyme][or:escherichia
coli][sr:**e**.coli (strain k12 ca274) dna, clones 12h5 and 4b10][db:
genpept-bct][dc:c.coli adk, visa genes and orfs.][nt:orf282][le:
1636][re:2478][di:complement]
14142188_f2_65 388 4042 198 66 145. . . [ac:f70019][pn:nifs protein homolog
homolog yurw][gn:yurw]
[or:bacillus subtilis][db:pir]
14336718_c3_199 402 4056 516 171 536 9.30E-52 [ln:efu09422][ac:u09422[[or:enter
ococcus faecalis][db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes, orfs 1-24, complete cds, complete sequence.]
[nt:orf18][le:4231][re:4728][di:direct]
14344510_f1_1 403 4057 849 282 1020 4.80E-103 [ln:ldgappgk][ac:aj000339][pn:gly
ceraldehyde-3-phosphate
dehydrogenase][gn:gap][or:lactobacillus delbrueckii][db:genpept-
bct][cc:1.2.1.12][de:lactobacillus. . . coli][de:phna protein]
[sp:p16680][db:swissprot]
1461636_f1_7 436 4090 678 225 180 4.90E-14 [ac:p37467][gn:xpac][or:bacillus
subtilis][de:xpac protein]
[sp:p37467][db:swissprot]
14626317_f1_2 437 4091 879 292 695 1.30E-68 [ln:spadca][ac:z71552][pn:hydrophob
ic **membrane** protein]
[gn:adcb][or:streptococcus pneumonia] [db:genpept-bct]
[de:streptococcus pneumonia adccba operon.][le:714][re:1517]
[di:direct]
14626562_c2_230 438 4092 990 329 835 1.90E-83 [ac:p54448][gn:yqec) [or:bacillus
subtilis][de:hypothetical 32.8 kd
. . .
DETD . . . cds, and na+/h+
antiporter(nhac), nahs (nahs), orfb, orfc, and orfd genes,
14735343_c1_16 470 4124 294 97 77 0.021 [ln:ae001158][ac:ac001158:ac000783][pn:
conserved hypothetical
integral **membrane**][gn:bh0574][or:borrelia burgdorferi][sr:lyme

disease spirochete][db:genpept bct][de:borrelia burgdorferi (section
44 of 70) of the complete genome.][nt:similar to pir:
14735817_f1_1 471 4125 789 262 425 5.40E-40. . . 774 257 72 0.063
[ac:p19281][or:thermoproteus tenax virus 1][sr:kra1,ttv1][de:
hypothetical 8.9 kd protein][sp:p19281][db:swissprot]
14882678_f3_24 484 4138 966 321 1303 4.90E-133 [ln:efplsep1g][ac:x96976][pn:tra
nsposase][gn:tnp1062][or:
enterococcus faecalis][db:genpept-bct][de:**e**.faccalis plasmid dna
sep1 gene, 4068bp.][le:2496][re:3455][di:complement]
14884712_c3_55 485 4139 1071 356 358 6.70E-33 [ac:s75550][pn:dtdp-glucose
4-6-dehydratase:protein s1r0809:
protein s1r0809][gn:rfbb][or:syncchocystis sp.][sr:pcc 6803, ,
pcc. . . [ac:q04535][gn:per][or:drosophila mediostriata][sr:,fruit
fly][de:
period clock protein (fragment)][sp:q04535][db:swissprot]
14959452_c1_119 490 4144 354 117 489 8.901E-47 [ln:cfu09422][ac:u09422][or:ente
rococcus faecalis][db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes, orfs 1-24, complete cds, complete sequence.][nt:
orf21][le:1081][re:2466][di:direct]
14970418_f3_6 491 4145 651 216 353 2.30E-32 [ac:p39145][gn:comfa:comfl][or:baci
llus subtilis][de:comf operon
protein. . . 4146 186 61 58 0.24 [ac:p39709][gn:sco1:ya1067c][or:sacc
haromyces cerevisiae][sr:
baker's yeast][de:sco1 protein][sp:p39709][db:swissprot]
15017013_f2_5 493 4147 876 291 752 1.20E-74 [ac:p42361][or:streptococcus
gordonii challis][de:29 kd **membrane**
protein in psaa 5'region (orf1)][sp:p42361][db:swissprot]
15017562_c2_26 494 4148 216 71 218 4.60E-18 [ln:ac001132][ac:ac001132:ae000783]
[pn:phosphate abc
transporter, atp-binding protein][gn:bb0218][or:borrelia
burgdorferi][sr:lyme disease spirochete][db:genpept-bct]
[de:borrelia. . . [ac:p90921][gn:k07a12.3][or:caenorhabditis
elegans][ec:3.6.1.34]
[de:putative atp synthase g chain, mitochondrial 2,][sp:p90921]
[db:swissprot]
15719390_f1_10 541 4195 195 64 66 0.55 [ac:p25201][or:acinetobacter
calcoaceticus][ec:2.1.1.72][de:
methyltransferase acci) (**m**.acci)][sp:p25201][db:swissprot]
15720716_f3_22 542 4196 894 297 175 4.80E-12 [ac:d69789][pn:hypothetical
protein ydjh][gn:ydjh][or:bacillus
subtilis][db:pir]
15728800_c1_16 543 4197 1281 426 1193 2.20E-121 [ac:p05648][gn:dnaa:dnah][or:ba
cillus subtilis][de:chromosomal
replication initiator. . . protein-npi-phosph
15899050_f3_23 565 4219 2148 715 1658 1.20E-170 [ac:c69810][pn:anion-binding
protein homolog yfle][gn:yfle][or:
bacillus subtilis][db:pir]
15899217_c1_28 566 4220 648 215 90 0.53 [ac:s51869][pn:probable **membrane**
protein ydr141c:hypothetical
protein yd9302.17c][or:saccharomyces cerevisiae][db:pir][mp:4r]
15901552_c2_61 567 4221 201 66 67 0.071 [ac:p35706][gn:sti2][or:streptomyces
longisporus][de:trypsin
inhibitor sti2 precursor][sp:p35706][db:swissprot]
15909808_c3_4 568 4222 519 172. . .
DETD . . . transferase.][nt:weak homology with vsf-1 gene (x73635)][le:
16197587_c3_198 583 4237 273 90 296 2.50E-26 [ln:efu09422][ac:u09422][or:entero
coccus faecalis][db:genpept bct]
[de:enterococcus faccalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes, orfs 1-24, complete cds, complete sequence.][nt:
orf19][le:3893][re:4114][di:direct]
16210375_c2_122 584 4238 1011 336 330 6.30E-30 [ac:d69588][pn:transcriptional
repressor of the. . . [db:pir]
16406327_c2_163 591 4245 1071 356 208 1.30E-14 [ac:q50735][gn:mtcy9c4.05c][or:m
ycobacterium tuberculosis][de:
hypothetical 40.2 kd protein cy9c4.05c][sp:q50735][db:swissprot]

aries:capra hircus][sr:,sheep:
goat][de:sperm protamine p1 (cysteine-rich protamine)][sp:p04102]
[db:swissprot]
16410200_c2_57 593 4247 885 294 664 2.50E-65 [ac:d69759][pn:hypothetical
protein ycgq][gn:ycgq][or:bacillus
subtilis][db:pir]
16411604_c1_26 594 4248. . .
DETD 681 4335 2784 927 1119 5.90E-120 [ac:p23914][gn:levr][or:bacillus
subtilis][de:transcriptional
regulatory protein levr][sp:p23914][db:swissprot]
16990753_c2_64 682 4336 216 71 63 0.12 [ac:a61429][pn:**m** protein pepm57][cl:m5
protein][or:streptococcus
pyogenes][db:pir]
16995162_c1_18 683 4337 192 63 46 0.25 [ac:p09639][gn:atpa][or:sulfolobus
acidocaldarius][ec:3.6.1.34]
[de:alpha)][sp:p09639][db:swissprot]
16995162_c1_5 684 4338 192 63 46 0.25 [ac:p09639][gn:atpa][or:sulfolobus. . .
447 148 256 4.30E-22 [ac:p44558][gn:hi0186][or:haemophilus
influenzae][de:hypothetical
transcriptional regulator hi0186][sp:p44558][db:swissprot]
172277_f1_15 707 4361 201 66 76 0.041 [ac:p52371][gn:gm:39][or:equine
herpesvirus type 2][sr:86/87,
ehv-2][de:glycoprotein **m**][sp:p52371][db:swissprot]
172281_c1_22 708 4362 1266 421 868 6.10E-87 [ac:d69596][pn:branched-chain amino
acid transporter brnq][gn:
brnq][or:bacillus subtilis][db:pir]
172558_f2_21 709 4363 195 64 59 0.49 [ac:p17175][gn:u14][or:human. . .
[gn:ybbt][or:bacillus subtilis][db:pir]
194093_c1_27 715 4369 354 117 413 1.00E-38 [ac:s68609][pn:recombinase
sin][or:staphylococcus aureus]
[db:pir]
194452_f3_21 716 4370 258 85 90 0.00036 [ln:bbu80959][ac:u80959:178251][pn:puta
tive outer **membrane**
protein][gn:ospfi][or:borrelia burgdorferi][sr:lyme disease
spirochete][db:genpept-bct][de:borrelia burgdorferi strain n40ch
putative outer **membrane** protein(ospfi) gene, complete cds.][nt:
19531500_f3_17 717 4371 210 69 69 0.058 [ln:cmu23045][ac:u23045][pn:nadh
dehydrogenase subunit 6][or:
mitochondrion cepaea nemoralis][sr:banded wood snail][db:
genpept-inv][de:cepaea. . . 749 4403 345 114 414 7.80E-39
[ac:g69633][pn:glutamine abc transporter (atp-binding protein)
glnq][gn:glnq][or:bacillus subtilis][db:pir]
19631430_f2_10 750 4404 210 69 72 0.39 [ac:jc6009][pn:surface-locatcd
membrane protein lmp3][gn:lmp3]
[or:mycoplasma hominis][db:pir]
1964457_c3_22 751 4405 237 78 65 0.073 [ac:p05768][or:halobacterium
halobium:halobacterium cutirubrum]
[de:50s ribosomal protein 112 (`a` type) (h120)][sp:p05768]
. . . .
DETD . . . lar][sr:,common gibbon]
[ec:3.6.1.34][de:atp synthase protein 8, (a61)][sp:q95705]
[db:swissprot
20161377_f1_2 806 4460 204 67 53 0.76 [ac:c21774][pn:el
glycoprotein][cl:togavirus structural polyprotein]
[or:venezuelan equine **encephalitis virus**][db:pir]
20167818_c1_30 807 4461 204 67 70 0.022 [ln:af034606][ac:af034606][pn:chordin][
or:danio rerio][sr:
zebrafish][db:genpept-vrt][de:danio rerio chordin mrna, complete
cds.][le:152][re:2974][di:direct]
20179682_c2_39 808 4462 468 155 533 1.90E-51. . . subunit (rpoc),putative
dna binding protein,
putative abc transporter subunitcomya (comya), putative abc
transport
2041678_f2_18 831 4485 633 210 102 0.019 [ac:s59310][pn:probable **membrane**
protein ymr317w:hypothetical

protein ym9924.09][or:saccharomyces cerevisiae][db:pir][mp:15r]
204717_c2_156 832 4486 402 133 461 8.20E-44 [ln:efu09422][ac:u09422][or:enteroc
occus faecalis][db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes, orfs 1-24, complete cds, complete sequence.][nt:
orf17][le:4703][re:5209][di:direct]
2048162_f2_4 833 4487 912 303 360 5.60E-33 [ac:a70009][pn:two-component sensor
histidine kinase. . . hirae][db:pir]
20573500_c2_234 852 4506 300 99 287 2.30E-25 [ac:p96349][gn:cspl][or:lactobacil
lus plantarum][de:cold shock
protein 2][sp:p96349][db:swissprot]
20585943_c2_9 853 4507 729 242 954 4.70E-96 [ln:efplsep1g][ac:x96976][pn:transp
osase][gn:tnp1062][or:
enterococcus faecalis][db:genpept-bct][de:**e**.faecalis plasmid dna
sep1 gene, 4068bp.][le:2496][re:3455][di:complement]
20585943_c3_10 854 4508 729 242 955 3.70E-96 [ln:efplsep1g][ac:x96976][pn:trans
posase][gn:tnp1062][or:
enterococcus faecalis][db:genpept-bct][de:**e**.faecalis plasmid dna
sep1 gene, 4068bp.][le:2496][re:3455][di:complement]
20586592_c3_50 855 4509 912 303 321 5.60E-29 [ln:llu36837][ac:u36837][pn:abicii
][fn:with abici, abortive infection
bacteriophage][or:lactococcus lactis][db:genpept-bct][de:
lactococcus lactis plasmid. . . 6.00E-112
[ln:111pk214][ac:x92946:y10522][pn:macrolide efflux protein][gn:
mef214][or:lactococcus lactis][db:genpept-bct][de:lactobacillus
lactis plasmid pk214, complete sequence.][le:10534][re:11790]
[di:direct]
20978375_f2_20 881 4535 402 133 128 1.60E-08 [ln:ececoprri][ac:x98141][gn:doc][
or:escherichia coli][db:
genpept-bct][de:**e**.coli dna sequence upstream of the ecoprri hsd
locus.][le:401][re:781][di:direct]
20992205_f2_19 882 4536 735 244 98 0.028 [ac:q05813][gn:peppi:pepp][or:streptom
yces lividans][ec:3.4.11.9]
[de:i)][sp:q05813][db:swissprot]
20992925_f3_59 883 4537 2628. . .
DETD . . . 195 64 58 0.34 [ln:d90883] [ac:d90883:ab001340] [pn:csie
protein.] [gn:csie] [or:
escherichia coli]
[sr:escherichia coli (strain:k12) dna, clone_lib:kohara
lambda minise] [db:genpept-bct]
[de:**e**.coli genomic dna, kohara clone #430(57.2-57.5 min.).]
[nt:similar to [swisspr
21656655_f2_49 922 4576 231 76 66 0.063 [ln:pop29g14] [ac:x04962]
[or:bacteriophage phi-29]
[db:genpept-phg]. . . 252 7.00E-21 [ln:ab007465] [ac:ab007465]
[pn:dna gyrase
subunit a] [gn:gyra coding region encoding for
dna gyrase subunit] [or:streptococcus thermophilus]
[sr:streptococcus thermophilus (strain:**m**-
192) dna] [db:genpept-bct] [de:streptococcus thermophilus gene for dna g
21673127_c1_25 926 4580 870 289 302 5.80E-27 [ac:c70066] [pn:conserved
hypothetical protein ywpj]. . . 471 208 7.80E-15 [ac:q58902]
[gn:mj1507] [or:methanococcus jannaschii]
[de:hypothetical protein mj1507]
[sp:q58902] [db:swissprot]
22064843_f1_17 976 4630 186 61 46 0.52 [ac:s70114] [pn:probable **membrane**
protein ydr284c:hypothetical
protein d9819.10] [gn:dpp1]
[or:saccharomyces cerevisiae] [db:pir] [mp:4r]
22070443_c1_87 977 4631 2553 850 562 9.70E-81 [ac:p23914] [gn:levr]
[or:bacillus subtilis][de:transcriptional regulatory. . . 1129
1.30E-114 [ac:s68603:s45077:s45078] [pn:hypothetical protein gamma]
[gn:gamma] [or:streptococcus pyogenes] [db:pir]
22150426_c2_58 983 4637 849 282 365 1.20E-33 [ac:p08188] [gn:manz:ptsm:gptb]
[or:escherichia coli]
[de:(eii-**m**-man)] [sp:p08188]

[db:swissprot]
22152182_c1_38 984 4638 1587 528 1487 1.60E-152 [ac:q06752] [gn:cyss:spna]
[or:bacillus subtilis] [ec:6.1.1.16]
[de:(cysrs)] [sp:q06752]
[db:swissprot]
22164001_c3_75 985 4639 204 67. . .
DETD . . . yrre]
[gn:yrre] [or:bacillus subtilis]
[db:pir]
22462752_f2_6 1026 4680 201 66 359 5.30E-33 [ln:efentaorf] [ac:x94181]
[pn:enterocin a]
[gn:enta] [or:enterococcus faecium] [db:genpept-
bct] [de:**e**.faecium enta and orf2 genes.]
[le:108] [re:305] [di:direct]
22463937_f2_28 1027 4681 915 304 1028 6.80E-104 [ac:g69989] [pn:abc transporter
(permease) homolog ytcp]
[gn:ytcp]. . . [db:swissprot]
22832943_c3_76 1064 4718 231 76 61 0.18 [ln:d90845] [ac:d90845:ab001340]
[gn:yega] [or:escherichia coli]
[sr:escherichia coli
(strain:k12) dna, clone_lib:kohara lambda minise]
[db:genpept-bct] [de:**e**.coli genomic dna,
kohara clone #356(46.1-46.5 min.).] [nt:orf_id:o355#3; similar to
[swissprot a
22836088_c2_99 1065 4719 894 297 1044 1.40E-105 [ac:q54713] [gn:hasc]
[or:streptococcus. . . 86 0.0079 [ac:s40926] [pn:hypothetical protein
zk1098.3]
[or:caenorhabditis elegans] [db:pir]
22837756_f1_6 1067 4721 579 192 513 2.50E-49 [ac:d69670] [pn:glycine
betaine/carnitine/choline
abc transporter (**membrane** p) opucb]
[gn:opucb] [or:bacillus subtilis] [db:pir]
22844077_c1_121 1068 4722 1668 555 1755 6.20E-181 [ac:q59905] [gn:dexb]
[or:streptococcus equisimilis]
[ec:3.2.1.70] [de:(exo-1,6-alpha-
glucosidase) (glucodextranase)]. . .
DETD . . . cell function-associated antigen]
[gn:mafa] [or:homo
sapiens] [sr:human] [db:genpept-pri2] [de:homo sapiens mast
cell function-associated antigen
(mafa) mrna,complete cds.] [nt:type ii integral **membrane** glycoprotein; s
23569567_f3_32 1132 4786 210 69 68 0.22 [ac:p47544] [gn:mg302] [or:mycoplasma
genitalium]
[de:hypothetical protein mg302]
[sp:p47544] [db:swissprot]
235715_f2_2 1133 4787 522. . . [gn:vmp23] [or:borrelia hermsii]
[sr:borrelia hermsii strain=hs1; atcc 35209]
[db:genpept-bct] [de:borrelia hermsii variable
major protein 23 (vmp23) gene, completecds.] [nt:vmp23; outer **membrane**
li
23609711_c1_42 1147 4801 183 60 46 0.27 [ac:p18975] [or:panthera leo]
[sr:,lion] [de:hemoglobin alpha chain]
[sp:p18975] [db:swissprot]
23614665_c3_66 1148 4802 267 88. . . 532 2.50E-51 [ln:bacrplp] [ac:147971]
[pn:ribosomal protein s8] [gn:rpsh]
[or:bacillus subtilis] [db:genpept-
bct] [de:bacillus subtilis ribosomal protein
(rplpnxefroq, rpmedj,rpsqnhemk) genes, integral
membrane protein (secy) gene, adenylatekinase (adk) gene, methioni
2375253_c1_46 1204 4858 1629 542 1226 7.10E-125 [ac:a69584] [pn:alanyl-trna
synthetase alas] [gn:alas]
[or:bacillus subtilis]. . . [db:genpept-pln]
[de:chlorella vulgaris c-27 chloroplast dna, complete
sequence.] [nt:orf67] [le:53481] [re:53684] [di:direct]
23866453_f3_33 1228 4882 186 61 59 0.28 [ln:hiv1u13456] [ac:u13456]
[pn:**envelope** glycoprotein v1v2 region]

[gn:env] [or:human
immunodeficiency virus type 1] [db:genpept-vr1]
[de:human immunodeficiency virus type 1
isolate 024 from rwanda,**envelope** glycoprotein (env) gene, v1v2 region,
partial cd
23867311_c1_32 1229 4883 258 85 135 2.90E-09 [ac:q01620] [gn:jag] [or:bacillus
subtilis]
[de:jag protein (spoiiij. . .
DETD . . . [ac:p44550] [gn:hi0172] [or:haemophilus influenzae]
[de:hypothetical lipoprotein hi0172
precursor] [sp:p44550] [db:swissprot]
24000262_c2_63 1266 4920 768 255 226 6.60E-19 [ac:c64686] [pn:conserved
hypothetical integral
membrane protein hp1331] [or:heliobacter
pylori] [db:pir]
24007763_f1_8 1267 4921 348 115 76 0.37 [ln:mbu57538] [ac:u57538]
[pn:rhodopsin]
[or:myripristis berndti] [db:genpept-vrt]
[de:myripristis berndti rhodopsin. . . olpa gene.] [le:27]
[re:830] [di:direct]
24032842_f2_4 1284 4938 2070 689 3426 0 [ln:efpbp5g] [ac:x92687]
[pn:penicillin-binding protein 5]
[gn:pbp5] [or:enterococcus faecium]
[db:genpept-bct] [de:**e**.faecium pbp5 gene.] [le:127] [re:2163]
[di:direct]
24033562_c2_49 1285 4939 996 331 331 2.20E-36 [ac:s76964] [pn:hypothetical
protein]
[or:synechocystis sp.] [sr:pcc 6803, , pcc. . . utilis]
[ec:1.7.3.3] [de:uricase, (urate oxidase)]
[sp:p78609] [db:swissprot]
24064712_f3_54 1292 4946 1464 487 435 4.70E-41 [ac:p07908] [gn:dnab]
[or:bacillus subtilis]
[de:replication initiation and **membrane** attachment
protein] [sp:p07908] [db:swissprot]
24068966_f3_24 1293 4947 1005 334 486 1.80E-46 [ac:p30363] [gn:ansa]
[or:bacillus licheniformis]
[ec:3.5.1.1] [de:l-asparaginase, (l-asparagine
amidohydrolase)] [sp:p30363] [db:swissprot]
24081436_f3_31. . . (p23)] [sp:p39742] [db:swissprot]
24257712_c1_59 1328 4982 504 167 566 6.10E-55 [ln:charpqtou] [ac:z50854]
[pn:arpu]
[gn:arpu] [fn:muramidase-2 processing; autolysin
regulatory] [or:enterococcus hirae] [db:genpept-bct]
[de:**e**.hirae arp[q,r,s,t,u] genes.] [le:1230]
[re:1643] [di:direct]
24257802_c3_42 1329 4983 765 254 827 1.30E-82 [ac:p55339] [gn:ecsa:prst]
[or:bacillus subtilis]
[de:abc-type transporter atp-binding protein
ecsa]. . . 1112 8.50E-113 [ln:ab007465] [ac:ab007465] [pn:dna gyrase
subunit a]
[gn:gyra coding region encoding for
dna gyrase subunit] [or:streptococcus thermophilus]
[sr:streptococcus thermophilus (strain:**m**-
192) dna] [db:genpept-bct] [de:streptococcus thermophilus gene for dna g
24265755_f2_9 1336 4990 195 64 59 0.28 [ln:celf19b10] [ac:af000261]
[gn:f19b10.6]
[or:caenorhabditis elegans]. . .
DETD . . . pylori] [db:pir]
24305181_f3_48 1351 5005 399 132 251 1.50E-21 [ln:msgtewpa] [ac:m15467]
[pn:unknown protein]
[or:mycobacterium tuberculosis]
[sr:mycobacterium tuberculosis (strain erdman) dna] [db:genpept-bct]
[de:**m**.tuberculosis 65
kda antigen (cell wall protein a) gene.] [nt:orf f175; putative]
[le:242] [re:769] [d
24307952_f2_14 1352 5006 264 87 66 0.28. . . [ac:q11046] [gn:mtcy50.09]

[or:mycobacterium tuberculosis]
[de:hypothetical abc transporter
atp-binding protein cy50.09] [sp:q11046] [db:swissprot]
24397126_c3_83 1388 5042 243 80 60 0.23 [ln:ssgm643] [ac:x79528] [pn:**m**(-like)
protein]
[gn:emm lg643] [or:group g streptococcus]
[db:genpept-bct] [de:streptococcus sp. group g emm lg643 gene.]
[nt:type f] [le:<1] [re:
24397686_f2_12 1389 5043. . . 263 3.40E-19 [ln:u93872] [ac:u93872]
[or:kaposi's sarcoma-associated herpesvirus]
[sr:kaposi's sarcoma-
associated herpesvirus - human herpesvirus 8]
[db:genpept-vr1] [de:kaposi's sarcoma-associated
herpesvirus glycoprotein **m**, dnareplication protein, glycoprotein, dna
24408500_f1_4 1414 5068 552 183 78 0.15 [ln:af025396] [ac:af025396]
[gn:orf15x3] [or:vibrio anguillarum]
[db:genpept-bct] [de:vibrio
anguillarum rfb region,. . . [pn:reverse transcriptase (copia-like
retrotransposon)]
[or:liriodendron chinense]
[db:pir]
24412582_c1_170 1420 5074 528 175 613 6.40E-60 [ln:efplsep1g] [ac:x96976]
[pn:transposase] [gn:tnp1062]
[or:enterococcus faecalis]
[db:genpept-bct] [de:**e**.faecalis plasmid dna sep1 gene, 4068bp.]
[le:2496] [re:3455]
[di:complement]
24412902_c2_41 1421 5075 315 104 66 0.057 [ac:c69333] [pn:hypothetical protein
af0667] [or:archaeoglobus fulgidus]. . .
DETD . . . intergenic region] [sp:p07079] [db:swissprot]
24432937_c1_116 1485 5139 330 109 295 3.20E-26 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis]
[db:genpept-bct] [de:enterococcus
faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn) genes, orfs 1-24, complete cds,
complete sequence.] [nt:orf23] [le:336] [re:650] [di:direct]
24432952_c3_36 1486 5140 2145 714 321 1.60E-26 [ln:hvu95372] [ac:u95372]. . .
1020 339 1533 2.10E-157 [ac:f64626] [pn:gmp reductase] [or:helicobacter
pyfori] [db:pir]
24470375_f3_24 1491 5145 261 86 72 0.039 [ln:ecorhsd] [ac:m29719]
[or:escherichia coli] [sr:**e**.coli
(k12) cell line ch1330 dna, clone
pas3122] [db:genpept-bct] [de:c.coli rhsd gene encoding rshd protein,
3' end.] [nt:rhsd protein]
[le:<1]. . . subtilis] [ec:3.2.1.86]
[de:probable 6-phospho-beta
glucosidase,] [sp:p54716] [db:swissprot]
24486693_c2_71 1504 5158 309 102 477 1.70E-45 [ln:eharpqtou] [ac:z50854]
[pn:arpt] [gn:arpt]
[or:enterococcus hirae] [db:genpept-bct]
[de:**e**.hirae arp[q,r,s,t,u] genes.] [le:857] [re:1153] [di:direct]
24487812_c2_156 1505 5159 462 153 119 1.40E-07 [ln:bc332ab] [ac:y09323]
[pn:hypothetical protein] [gn:332b]
[fn:unknown] [or:bacillus cereus]
[db:genpept-bct]. . . i)]
[sp:p56296] [db:swissprot]
24500300_c2_68 1524 5178 225 74 69 0.13 [ln:lpatovgns] [ac:x94434] [pn:plni]
[gn:plni]
[fn:immunity protein] [or:lactobacillus
plantarum] [db:genpept-bct] [de:l.plantarum
pln[a,b,c,d,**e**,f,g,h,i,j,k,l,**m**,n,o,p,r,s,t,u,v] genesand
orf1.] [nt:putative] [le:9372] [re:10145] [di:complement]
24500962_c1_61 1525 5179 1065 354 577 4.20E-56 [ac:a69991] [pn:conserved
hypothetical protein yter] [gn:yter]
[or:bacillus subtilis]. . . 5199 660 219 572 1.40E-55 [ln:ecouw82]
[ac:110328] [gn:f270] [fn:unknown]

[or:escherichia coli] [sr:escherichia coli k12
strain mg1655; lambda clones ec14-52] [db:genpept-bct]
[de:e. coli; the region from 81.5 to
84.5 minutes.] [le:65538] [re:66350] [di:complement]
24620461_f1_8 1546 5200 2205 734 1994 2.90E-206 [ac:p28903] [gn:nrdd]
[or:escherichia. . . 125 49 1 [ln:styflga] [ac:d25292] [pn:flgb
protein] [gn:flgb] [fn:rod protein]
[or:salmonella typhimurium] [sr:salmonella typhimurium (strain:lt2) dna]
[db:genpept-bct] [de:salmonella
typhimurium flg (a,b,**m**,n) and orf (2,3) genes forflagella.]
[nt:author-given pro
24634687_c1_74 1549 5203 642 213 721 2.30E-71 [ac:p47848] [gn:tdk]
[or:streptococcus gordonii challis]
[ec:2.7.1.21] [de:thymidine. . . aureus]
[ec:4.1.--.--] [de:tagatose 1,6-diphosphate
aldolase,] [sp:p11100] [db:swissprot]
24642162_c1_46 1559 5213 561 186 232 1.50E-19 [ac:p43641] [gn:munim]
[or:mycoplasma sp] [ec:2.1.1.72]
[de:methyltransferase muni)
(**m**.muni)] [sp:p43641] [db:swissprot]
24642333_c2_60 1560 5214 618 205 72 0.78 [ln:ssz82017] [ac:z82017] [gn:unknown]
[or:sus scrofa] [sr:pig]
[db:genpept-est7] [de:s.scrofa
mrna; expressed sequence tag. . .
DETD . . . (oprt) (oprtase)] [sp:p25972] [db:swissprot]
24648438_c3_201 1568 5222 1011 336 1112 8.50E-113 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis]
[db:genpept-bct] [de:enterococcus
faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),(int-tn) genes,
orfs 1-24, complete cds,
complete sequence.] [nt:orf14] [le:9816] [re:10817] [di:direct]
24648438_f2_36 1569 5223 768 255 151 6.00E-09 [ac:p39842] [gn:bltr:bmtr:bmr2r].
. . kd
protein in ppa-fbp intergenic region
precursor] [sp:p39325] [db:swissprot]
24664217_f3_36 1586 5240 678 225 715 1.00E-70 [ac:f69670] [pn:glycine
betaine/carnitine/choline abc
transporter (**membrane** p) opucd]
[gn:opucd] [or:bacillus subtilis] [db:pir]
24664692_c3_68 1587 5241 3729 1242 2078 3.70E-215 [ac:p23478] [gn:adda]
[or:bacillus subtilis]
[de:atp-dependent nuclease subunit a]. . . subtilis]
[de:hypothetical 45.4 kd protein in sspb-prsa
intergenic region] [sp:o07523] [db:swissprot]
24667250_c2_20 1593 5247 1431 476 661 5.30E-65 [ac:d69159] [pn:methyl coenzyme
m reductase system, component a2 homolog]
[gn:mth454]
[or:methanobacterium thermoautotrophicum] [db:pir]
24667313_c2_104 1594 5248 1695 564 1215 1.00E-123 [ac:h69884] [pn:conserved
hypothetical protein ymfa]. . . protein vanrb] [sp:q47744]
[db:swissprot]
24804838_c1_117 1633 5287 387 128 376 8.30E-35 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis]
[db:genpept-bct] [de:enterococcus
faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),(int-tn) genes,
orfs 1-24, complete cds,
complete sequence.] [nt:orf22] [le:666] [re:1052] [di:direct]
24805312_c3_32 1634 5288 1887 628 1499 8.30E-154 [ln:cloabg] [ac:149336]. . .
DETD . . . [ac:f69694] [pn:ribosomal protein 12 (b12) rplb] [gn:rplb]
[or:bacillus subtilis] [db:pir]
24901712_c3_71 1686 5340 705 234 583 9.70E-57 [ac:f69633] [pn:glutamine abc
transporter (**membrane** protein) glnp]
[gn:glnp] [or:bacillus subtilis] [db:pir]
25058_f1_5 1687 5341 507 168 252 1.20E-21 [ac:c69874] [pn:conserved
hypothetical protein ylbf] [gn:ylbf]
[or:bacillus subtilis]. . . tentans (clone p62) reverse

transcriptase and orf1 union tcl retrotransposon genes, complete cds.]
25390762_c3_7 1695 5349 285 94 54 0.67 [ln:siu05093] [ac:u05093] [pn:**envelope** glycoprotein]
[gn:env] [or:simian immunodeficiency
virus] [db:genpept-vr1] [de:simian immunodeficiency virus sivrhe543 clone 4-20
envelopeglycoprotein (env) gene, v1 region, partial cds.] [nt:v1. . .
[or:bacteriophage phig1e] [db:genpept-phg]
[de:lactobacillus bacteriophage phig1e complete genomic dna.]
[le:4579] [re:5277]
[di:complement]
2542943_f3_18 1709 5363 1521 506 68 0.36 [ln:hivu79952] [ac:u79952]
[pn:**envelope** glycoprotein] [gn:env]
[or:human immunodeficiency
virus type 1] [db:genpept-vr1] [de:hiv-1 clone p6
ln 14v from uk, **envelope** glycoprotein,
v1/v2hypervariable region (env) gene, partial cds.] [nt:gp120; v1/v2 hyperva
25429640_c1_36 1710 5364 192 63 277 2.60E-24 [ac:jc5007] [pn:transposase
(insertion sequence. . . vulgaris c-27 chloroplast dna, complete sequence.] [nt:orf49b] [le:45442] [re:45591] [di:direct]
25442187_c1_61 1717 5371 1422 473 346 2.60E-57 [ac:g64666] [pn:conserved hypothetical integral
membrane protein hp1175] [or:helicobacter
pylori] [db:pir]
25445786_c3_45 1718 5372 588 195 498 9.80E-48 [ac:p32813] [or:bacillus stearothermophilus]
[de:hypothetical 18.2 kd protein in glda. . . [de:rho1 gdp-gtp
exchange protein 2] [sp:p51862] [db:swissprot]
25500312_c1_25 1725 5379 246 81 66 0.057 [ln:charpqtou] [ac:z50854] [pn:arpt]
[gn:arpt]
[or:enterococcus hirae] [db:genpept-bct]
[de:**e**.hirae arp[q,r,s,t,u] genes.] [le:857] [re:1153] [di:direct]
25507801_c2_42 1726 5380 378 125 269 1.80E-23 [ac:p32731] [gn:rbfa]
[or:bacillus subtilis]
[de:ribosome-binding factor a (p15b protein)]
. . . [gn:yesl]
[or:bacillus subtilis] [db:pir]
25587817_f2_41 1751 5405 1218 405 1032 2.50E-104 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis]
[db:genpept-bct] [de:enterococcus
faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn) genes, orfs1-24, complete cds,
complete sequence.] [nt:orf20] [le:2861] [re:3850] [di:direct]
25594751_c3_52 1752 5406 201 67 72 0.16 [ln:hsu79745] [ac:u79745]
[pn:monocarboxylate. . . gene] [le:245] [re:889] [di:direct]
25596051_c2_57 1754 5408 645 214 248 3.10E-21 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis]
[db:genpept-bct] [de:enterococcus
faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),(int-tn) genes,
orfs 1-24, complete cds,
complete sequence.] [nt:orf14] [le:9816] [re:10817] [di:direct]
25601562_c1_27 1755 5409 270 89 56 0.49 [ln:musptkd] [ac:m33424]. . . 246 81 69 0.028 [ac:d69773] [pn:hypothetical protein ydef] [gn:ydef]
[or:bacillus subtilis] [db:pir]
25789092_c1_7 1779 5433 201 66 63 0.12 [ln:hiv1u37039] [ac:u37039]
[pn:**envelope** glycoprotein] [gn:env] [or:human
immunodeficiency virus type 1] [db:genpept-vr1]
[de:human immunodeficiency virus type 1
isolate ar33 envelopeglycoprotein (env) gene, partial cds.]. . .
DETD . . . ii cosmid c3d5.] [nt:spbc3d5.14c,
unknown; partial; serine rich,] [le:31398] [re:
26220058_c3_70 1834 5488 489 162 548 5.00E-53 [ac:e69633] [pn:glutamine abc transporter
(**membrane** protein) glnm] [gn:glnm] [or:bacillus
subtilis] [db:pir]

[ac:p37466] [gn:veg] [or:bacillus
subtilis] [de:veg protein]
[sp:p37466] [db:swissprot]
26254806_c3_135. . .
DETD . . . 538 86 0.011 [ln:af019983] [ac:af019983] [gn:r1062]
[or:dictyostelium discoideum]
[db:genpept-inv] [de:dictyostelium discoideum r1062 gene, partial cds.]
[nt:similar to s. cerevisiae probable **membrane** protein] [le:<1]
[re:1655] [di:direct]
26594051_c2_97 1910 5564 1653 550 1882 2.20E-194 [ln:kpu95087] [ac:u95087]
[pn:mdca] [gn:mdca] [or:klebsiella pneumoniae]
[db:genpept-bct] [de:klebsiella pneumoniae malonate. . . [ac:s66773]
[pn:hypothetical protein yo1080c:hypothetical protein o1101]
[or:saccharomyces cerevisiae] [db:pir] [mp:151]
26804838_c1_180 1977 5631 870 289 315 2.40E-28 [ac:p08188] [gn:manz:ptsm:gptb]
[or:escherichia coli] [de:(eii-**m**-man)]
[sp:p08188] [db:swissprot]
26808437_c3_26 1978 5632 753 250 92 0.048 [ln:cmblab] [ac:x96858]
[pn:beta-lactamase] [gn:blab] [or:chryseobacterium
meningosepticum] [db:genpept-una] [ec:3.5.2.6] [de:c.meningosepticum
blab
gene.] [le:114]. . . [or:streptococcus thermophilus]
[db:genpept-bct] [de:s.thermophilus iss1sa dna for transposase.]
[le:113] [re:793]
[di:direct]
26822142_c1_8 1981 5635 909 302 258 2.70E-22 [ln:llpflmg13] [ac:aj000325]
[pn:putative **membrane** protein] [gn:orfa]
[or:lactococcus lactis] [db:genpept-bct] [de:lactococcus lactis pfl gene
(strain mg1363).] [le:270] [re:1187] [di:direct]
26828200_c3_66 1982 5636 225 74 68 0.015. . . 65 258 2.70E-22 [ac:d69701]
[pn:ribosomal protein s21 rpsu] [gn:rpsu] [or:bacillus subtilis]
[db:pir]
26839637_c2_71 1988 5642 882 293 575 6.80E-56 [ac:a56641] [pn:probable
membrane transport protein] [or:clostridium
perfringens] [db:pir]
26839688_f3_27 1989 5643 453 150 613 6.40E-60 [ac:p14577] [gn:rplp]
[or:bacillus subtilis] [de:50s ribosomal protein 116]
[sp:p14577]. . .
DETD . . . [db:genpept-inv] [de:caenorhabditis elegans non-muscle myosin
heavy chain ii (nmy-2)mrna, complete cds.] [
2912675_f2_2 2011 5665 189 62 57 0.57 [ac:s64940] [pn:probable **membrane**
protein ylr104w:hypothetical protein 12730]
[or:saccharomyces cerevisiae] [db:pir] [mp:12r]
2913885_f2_11 2012 5666 369 122
2928817_f3_9 2013 5667 2472 823 1936 4.30E-206 [ac:q04707] [gn:pona:exp2]. .
. [ln:mtv004] [ac:al009198] [pn:hypothetical protein mtv004.14]
[gn:mtv004.14]
[or:mycobacterium tuberculosis] [db:genpept-bct] [de:mycobacterium
tuberculosis sequence v004.] [nt:mtv004.14, unknown, len: 91 aa; similar
to
e. coli] [le:31376] [re:31651] [di:
29453433_f1_2 2054 5708 969 322 1009 7.00E-102 [ac:b70032] [pn:conserved
hypothetical protein yvcl] [gn:yvcl] [or:bacillus
subtilis] [db:pir]
2945452_c2_31 2055. . . cds.] [nt:orfb;
possible alternate start site at nt 686] [le:671] [re:1585] [di:direct]
29461541_c1_36 2058 5712 201 66 61 0.18 [ln:hivu67765] [ac:u67765]
[pn:**envelope** glycoprotein] [gn:env] [or:human
immunodeficiency virus type 1] [db:genpept-vrl] [de:hiv-1 isolate
tw334-1
from taiwan, **envelope** glycoprotein (env)gene, v3 region, partial cds.]
[nt:v3
region] [le:<1] [re:
29462555_c1_38 2059 5713 1941 646 260 6.70E-19 [ac:g69801] [pn:hypothetical
protein yfho]. . . 2101 5755 606 201 79 0.007 [ln:mmig11] [ac:v00764]
[pn:gamma 2a heavy chain of immunoglobulin g]

[or:mus musculus] [sr:house mouse] [db:genpept 10d] [de:m.musculus
mrna for immunoglobulin gamma 2a.] [le:<1] [re:
29922951_f2_9 2102 5756 654 217 207 6.80E-17 [ac:p15454]
[gn:guk1:ydr454c:d9461.39] [or:saccharomyces cerevisiae]
[sr:,baker's yeast] [ec:2.7.4.8]. . . 5760 309 102 57 0.86
[ln:bbbrgabcd] [ac:x87201] [gn:orfa] [or:borrelia burgdorferi] [sr:lyme
disease
spirochete] [db:genpept-bct] [de:b.burgdorferi plasmid, orfa, b, c, d,
e, & f genes, clone pomb14and pomb17.] [le:594] [re:1691] [di:direct]
29960041_c1_68 2107 5761 252 83 69 0.35 [ln:ataf002109] [ac:af002109]
[gn:t28m21.10] [or:arabidopsis thaliana]
. . .
DETD . . . transposase] [le:140] [re:1414] [di:direct]
3022781_c2_90 2131 5785 264 87 101 1.20E-05 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf5] [le:16121]
[re:16372] [di:complement]
30260916_c2_98 2132 5786 1701 566 694 1.70E-68 [ln:kpu95087] [ac:u95087]. . .
[ac:q48514] [gn:tnha] [or:leptospira borgpetersenii] [de:transposase for
insertion
sequence element is1533] [sp:q48514] [db:swissprot]
30519450_c2_141 2149 5803 198 65 54 0.67 [ac:s67565] [pn:probable **membrane**
protein ydl032w:hypothetical protein d2767]
[or:saccharomyces cerevisiae] [db:pir] [mp:41]
30553567_c2_44 2150 5804 522 173 407 4.30E-38 [ac:s39974] [pn:hypothetical
protein] [or:streptococcus equisimilis] [db:pir]
30557807_f1_3. . . translocase secy subunit] [sp:p27148] [db:swissprot]
30711077_f1_1 2164 5818 957 318 199 7.00E-16 [ac:p16055] [gn:tpp15]
[or:treponema pallidum] [de:15 kd lipoprotein precursor
(major **membrane** immunogen)] [sp:p16055] [db:swissprot]
30713162_f1_1 2165 5819 1017 338 279 1.60E-24 [ac:c69805] [pn:iron(iii)
dicitrate transport permease homolog yfiy] [gn:yfiy]
[or:bacillus subtilis] [db:pir]
30713265_f1_1 2166 5820 417 138 77 0.04 [ac:c64578] [pn:conserved hypothetical
integral **membrane** protein hp0467]
[or:helicobacter pylori] [db:pir]
30744010_c3_111 2167 5821 999 332 483 3.80E-46 [ln:af034088] [ac:af034088]
[pn:lipase] [gn:lipp] [or:pseudomonas sp.
b11-1] [db:genpept] [de:pseudomonas. . . 336 203 3.30E-16 [ac:g69986]
[pn:hypothetical protein ysnf] [gn:ysnf] [or:bacillus subtilis]
[db:pir]
3126542_f3_18 2177 5831 345 114 107 1.50E-05 [ln:scu77778] [ac:u77778:u29130]
[pn:putative **membrane** protein] [gn:epih]
[fn:involved in epidermin secretion] [or:staphylococcus epidermidis]
[db:genpept-bct] [de:staphylococcus epidermidis plasmid ptue32 putative
abc transportersubunits (epig), (epie),
3126562_f2_13 2178 5832. . . 681 226 403 1.10E-37 [ac:p54443] [gn:yrkp]
[or:bacillus subtilis] [de:intergenic region] [sp:p54443]
[db:swissprot]
31442937_c2_68 2197 5851 369 122 55 0.71 [ln:hivlu48123] [ac:u48123]
[pn:**envelope** glycoprotein, c2-v5 region] [gn:env]
[or:human immunodeficiency virus type 1] [db:genpept-vr1] [de:human
immunodeficiency virus type 1 clone 62s sample i envelopeglycoprotein.
. . [gn:yqfr] [or:bacillus subtilis] [de:probable rna helicase in
ccca-soda
intergenic region] [sp:p54475] [db:swissprot]
31535081_c2_22 2206 5860 183 60 55 0.64 [ln:hivlu08822] [ac:u08822]
[pn:**envelope** glycoprotein, c2v3 region] [gn:env]
[or:human immunodeficiency virus type 1] [sr:human immunodeficiency
virus
type 1, sample 024 from uganda] [db:genpept-vr1] [de:human
. . . [or:saccharomyces cerevisiae]
[sr:,baker's yeast] [ec:6.1.1.19] [de:-trna ligase) (argrs)] [sp:q05506]
[db:swissprot]

[or:bacillus subtilis] [de:**membrane**-bound protein lytr]
[sp:q02115] [db:swissprot]
3204549_c1_28 2234 5888 303 100 120 1.20E-07 [ac:e69826] [pn:1-acylglycerol-3-phosphate o-acyltransfera homolog yhdo]
[gn:yhdo] [or:bacillus subtilis] [db:pir]
32047676_c2_13 2235. . .
DETD . . . shia and shib, putative abc transporter shic,
and putativepermeases shid
32213278_f2_11 2254 5908 186 61 102 9.00E-06 [ln:synorflac] [ac:m15619]
[or:artificial sequence] [sr:**e**.coli (strain se5000)
synthetic dna, clone pkb1] [db:genpept-syn] [de:synthetic **e**.coli
orf16/lacz
fusion protein, partial cds.] [nt:orf16-lacz fusion protein] [le:29]
[re:
32213278_f2_5 2255 5909 186 61 102 9.00E-06 [ln:synorflac] [ac:m15619]
[or:artificial sequence] [sr:**e**.coli (strain se5000)
synthetic dna, clone pkb1] [db:genpept-syn] [de:synthetic **e**.coli
orf16/lacz
fusion protein, partial cds.] [nt:orf16-lacz fusion protein] [le:29]
[re:
32218761_c1_2 2256 5910 183 60 61 0.18 [ac:s73440] [pn:hypothetical protein
b01_orf103b]. . . receptor|car [human,
neonatal foreskin, keratinocytes, mrnapartial, 798 nt].] [le:1] [re:798]
[di:direct]
32313_f1_6 2273 5927 225 74 68 0.072 [ln:eavrmn0] [ac:x78501] [pn:small
envelope protein] [gn:**m**] [or:equine arteritis
virus] [db:genpept-vrl] [de:equine arteritis virus (isolate norw2)
mrna for **m** and n proteins.] [le:8] [re:496] [di:direct]
32319831_c3_71 2274 5928 252 83 67 0.026 [ac:o05543] [or:gluconobacter
suboxydans] [de:hypothetical protein in adhs
5'region. . . [ac:i40055] [pn:positive trans-activator of capsule
synthesis] [gn:acpa]
[or:bacillus anthracis] [db:pir]
32788_c3_33 2292 5946 1263 420 666 1.60E-65 [ac:q02115] [gn:lytr] [or:bacillus
subtilis] [de:**membrane**-bound protein lytr]
[sp:q02115] [db:swissprot]
3298262_c1_120 2293 5947 387 128 344 2.10E-31 [ac:f69791] [pn:conserved
hypothetical protein yebb] [gn:yebb]
[or:bacillus subtilis] [db:pir]
3313510_c2_43 2294. . . [db:genpept-pri2] [de:homo sapiens atrophin-1
related protein mrna,
partial cds.] [nt:hatrp] [le:565] [re:
3317090_f2_19 2295 5949 240 79 60 0.23 [ac:s61055:s67706:s67710] [pn:probable
membrane protein yd1158c:hypothetical
protein d1530] [or:saccharomyces cerevisiae] [db:pir] [mp:41]
33203387_c2_14 2296 5950 228 75 66 0.063 [ac:p48934] [gn:sdh3:sdhc]
[or:chondrus crispus] [sr:,carragheen]
[de:dehydrogenase,. . . [gn:yusp]
[or:bacillus subtilis] [db:pir]
33605083_c1_124 2353 6007 2211 736 1785 4.10E-184 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf15] [le:7643]
[re:9907] [di:direct]
3360837_c3_142 2354 6008 294 97 74 0.053 [ln:apu96137]. . .
DETD . . . 1.10E-13 [ac:a69627] [pn:fructose 1-phosphate kinase frub]
[gn:frub] [or:bacillus subtilis]
[db:pir]
33673317_c1_6 2358 6012 786 261 525 3.80E-66 [ac:p08188] [gn:manz:ptsm:gptb]
[or:escherichia coli] [de:(eii-**m**-man)]
[sp:p08188] [db:swissprot]
33678557_f1_7 2359 6013 546 181 105 0.0019 [ac:p37198] [gn:nup62] [or:homo
sapiens] [sr:,human] [de:nuclear pore
glycoprotein p62 (nucleoporin p62)] [sp:p37198]. . . 8.40E-20

[ac:p32056] [gn:cmcb] [or:escherichia coli] [ec:2.7.1.65] [de:enzyme 11,
a component),] [sp:p32058] [db:swissprot]
33792553_c2_152 2375 6029 531 176 92 0.0082 [ac:i41076] [pn:methyltransferase
m.ecohk31i beta chain] [or:escherichia coli]
[db:pir]
33798177_f2_22 2376 6030 855 284 767 3.10E-76 [ac:e69742] [pn:abc transporter
(atp-binding protein) homolog ybae] [gn:ybae]
[or:bacillus. . . 132 482 4.90E-46 [ln:bacrplp] [ac:147971]
[pn:ribosomal protein s13] [gn:rpsm]
[or:bacillus subtilis] [db:genpept-bct] [de:bacillus subtilis
ribosomal protein (rplpnxefroq, rpmcdj,rpsqnhemk) genes, integral
membrane protein (secy)
gene, adenylatekinase (adk) gene, methion
34074027_c3_57 2419 6073 1257 418 834 2.40E-83 [ln:spdnagcpo] [ac:y11463]
[or:streptococcus pneumoniae] [db:genpept-bct]
[de:streptococcus pneumoniae. . . 327 108 59 0.28 [ac:d60396]
[pn:antigen 7h8/7] [cl:pol polyprotein] [or:plasmodium falciparum]
[db:pir]
34120463_c2_40 2428 6082 414 137 85 0.17 [ac:pc6003] [pn:surface **membrane**
protein lmp4:hypothetical 624
protein:lmp4 protein] [gn:lmp4] [or:mycoplasma hominis] [db:pir]
34157517_c3_8 2429 6083 966 321 774 5.60E-77 [ac:d69744] [pn:conserved
hypothetical protein ybbi]. . .
DETD . . . protein] [le:14319:14685:16003] [re:14462:14827:16162]
[di:directjoin]
34192183_c3_44 2476 6130 327 108 117 2.30E-07 [ln:soorfs] [ac:z79691]
[gn:yorfe] [fn:putative transcription regulator]
[or:streptococcus pneumoniae] [db:genpept-bct] [de:s.pneumoniae
yorf[a,b,c,d,**e**],
ftsl, pbpx and regr genes.] [le:2388] [re:2582] [di:complement]
34192187_c2_114 2477 6131 486 161 46 0.14 [ac:c69915] [pn:hypothetical protein
yont] [gn:yont] [or:bacillus. . . 117 1.60E-07 [ac:p44611]
[gn:hi0282] [or:haemophilus influenzae] [de:hypothetical protein
hi0282] [sp:p44611] [db:swissprot]
34195750_c2_239 2481 6135 969 322 259 2.10E-22 [ln:llpflmg13] [ac:aj000325]
[pn:putative **membrane** protein] [gn:orfa]
[or:lactococcus lactis] [db:genpept-bct] [de:lactococcus lactis pfl gene
(strain mg1363).] [le:270] [re:1187] [di:direct]
34195932_c1_125 2482 6136 858 285 179 1.90E-12. . . [db:genpept-bct]
[de:serratia marcescens
putative dtdp-4-dehydrorhamnose 3,5 epimerase(rmlc), putative
dtdp-1-rhamnose
synthase (rmld), putativerhamn
34573441_c1_29 2544 6198 1227 408 1047 6.60E-106 [ln:atu91632] [ac:u91632]
[pn:**membrane**-spanning permease] [gn:ggub]
[or:agrobacterium tumefaciens] [db:genpept-bct] [de:agrobacterium
tumefaciens
sugar transporter (ggua),**membrane**-spanning permease (ggub), and (gguc)
genes,
complete cds.] [le:1628] [re:28
34573461_f1_2 2545 6199 681 226 102 0.0037 [ln:liinlc] [ac:y07639] [gn:orf z]
[fn:putative. . . [db:swissprot]
34586528_c3_35 2560 6214 1548 515 1527 9.00E-157 [ln:atu91632] [ac:u91632]
[pn:sugar transporter] [gn:ggua] [or:agrobacterium
tumefaciens] [db:genpept-bct] [de:agrobacterium tumefaciens
sugar transporter (ggua), **membrane**-spanning permease (ggub), and
(gguc) genes,
complete cds.] [nt:atp-binding protein]
34589052_f3_3 2561 6215 960 319 311 6.40E-28 [ln:ab005215] [ac:ab005215]
[pn:301aa long hypothetical. . .
DETD . . . [nt:orf1] [le:638] [re:1213] [di:direct]
35188262_c3_195 2615 6269 1059 352 991 5.60E-100 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf21] [le:1081]

[re:2400] [di:direct]
35189757_c2_18 2616 6270 753 250 424 6.80E-40 [ac:g69766]. . . 2620 6274 921
306 320 7.20E-29 [ln:styflga] [ac:d25292] [pn:orf3] [gn:orf3]
[or:salmonella typhimurium]
[sr:salmonella typhimurium (strain:lt2) dna] [db:genpept-bct]
[de:salmonella typhimurium flg (a,b,**m**,n) and orf (2,3) genes
forflagella.]
[le:3746] [re:4669] [di:complement]
35204002_f3_34 2621 6275 225 74 182 3.00E-14 [ln:bsz75208] [ac:z75208]
[pn:hypothetical protein] [gn:ysoc] [or:bacillus. . . 131 5.60E-12
[ln:d90829] [ac:d90829:ab001340] [pn:isochorismatase (ec 3.3.2.1)
(2,3 dihydro-2,3] [gn:yecd] [or:escherichia coli] [sr:escherichia coli
(strain:k12) dna, clone_lib:kohara lambda minise] [db:genpept-bct]
[de:**e**.coli
genomic dna, kohara clone #337(41.9-42.3
35355340_f1_2 2639 6293 453 150 686 1.20E-67 [ln:chy13922] [ac:y13922:y15222]
[gn:yllb] [or:enterococcus hirae]
[db:genpept-bct] [de:enterococcus hirae mrar,. . . ftsq
and ftsagenes, mraw, yllc and ftsz partial genes.] [le:57] [re:503]
[di:direct]
35359755_f3_30 2640 6294 315 104 138 1.20E-08 [ac:s61993:s66874:s72142]
[pn:probable **membrane** protein yor009w:hypothetical
protein o2549:hypothetical protein unb487] [or:saccharomyces cerevisiae]
[db:pir] [mp:15r]
35363437_f2_73 2641 6295 195 64 67 0.53 [ln:af007261] [ac:af007261] [pn:haem
lyase]. . . [de:excinuclease abc subunit c]
[sp:p14951] [db:swissprot]
35579687_c3_35 2655 6309 1305 435 1548 5.30E-159 [ac:p21458:p21459]
[gn:spoiiie] [or:bacillus subtilis] [de:stage iii sporulation
protein **e**] [sp:p21458:p21459] [db:swissprot]
35584711_f1_7 2656 6310 759 252 516 1.20E-49 [ln:instranspo] [ac:134675]
[pn:transposase] [or:insertion sequence is1251]
[sr:insertion sequence is1251 dna] [db:genpept-bct] [de:insertion. . .
[ac:p41508] [or:mycoplasma hyorhinis] [de:p115 protein] [sp:p41508]
[db:swissprot]
35817167_c3_146 2673 6327 357 118 411 1.60E-38 [ln:charpqtou] [ac:z50854]
[pn:arpt] [gn:arpt] [or:enterococcus hirae]
[db:genpept-bct] [de:**e**.hirae arp[q,r,s,t,u] genes.] [le:857] [re:1153]
[di:direct]
35829712_c2_53 2674 6328 1137 378 57 0.99 [ln:mratef1b2] [ac:m16353]
[or:rhizomucor racemosus] [sr:**m**.racemosus
(atcc 1216b) germling dna] [db:genpept-pln] [de:**m**.racemosus elongation
factor 1-alpha (tef-2) gene, 3' end.] [nt:elongation factor tef-2]
[le:<1]
[re:140] [di:direct]
35839515_c1_37 2675 6329 1239 412 1058 4.50E-107 [ac:s37549:s67927]. . .
[or:pasteurella haemolytica] [db:genpept-bct] [de:pasteurella
haemolytica
exbb (exbb) exbd (exbd) and tonb (tonb)genes, complete cds and cysz
(cysz) gene, partial cds.] [nt:**membrane** prot
35990893_c1_120 2691 6345 198 65 78 0.024 [ln:celb0218] [ac:u58752]
[gn:b0218.5] [or:caenorhabditis elegans]
[sr:caenorhabditis elegans strain=bristol n2] [db:genpept-inv]
[de:caenorhabditis elegans cosmid. . .
DETD . . . [gn:yhan]
[or:bacillus subtilis] [db:pir]
36046965_f2_4 2700 6354 411 136 160 2.20E-11 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf20] [le:2861]
[re:3850] [di:direct]
36047176_f1_2 2701 6355 1035 344 468 1.50E-44 [ac:p15294] [gn:prtm]
[or:lactococcus lactis] [sr:,subsplactis:streptococcus
lactis] [de:protease maturation protein precursor] [sp:p15294]
[db:swissprot]

protein homolog] [or:archaeoglobus fulgidus] [db:pir]
36070317_c1_18 2703 6357 216 71 59 0.28 [ac:p24649] [or:bombyx mori nuclear
polyhedrosis virus] [sr:,bmnpv] [de:protein)
(nucleocapsid. . . [ac:e69751] [pn:abc transporter (atp-binding
protein) homolog ybxa] [gn:ybxa]
[or:bacillus subtilis] [db:pir]
36150187_c3_65 2723 6377 282 93 69 0.16 [ln:tbz56279] [ac:z56279] [pn:integral
membrane protein] [gn:cglf]
[or:thermoanaerobacter brockii] [db:genpept-bct] [de:t.brockii
cglf, cglg, xgls and cglt genes.] [le:191] [re:1102] [di:direct]
36187800_c3_64 2724 6378 627 208 253. . . [gn:repb] [or:enterococcus
faecalis]
[db:pir]
36210963_c3_202 2737 6391 930 309 692 2.70E-68 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf13]
[le:10814]
[re:11746] [di:direct]
36211013_f3_37 2738 6392 306 101 75 0.029 [ac:170091] [pn:hypothetical protein
yydh] [gn:yydh] [or:bacillus subtilis]
[db:pir]
36211556_c2_122 2739 6393 846 281 454 4.50E-43 [ac:p08188] [gn:manz:ptsm:gptb]
[or:escherichia coli] [de:(eii-**m**-man)]
[sp:p08188] [db:swissprot]
36214637_c2_85 2740 6394 951 316 1388 4.80E-142 [ac:q47741] [gn:pyrd]
[or:enterococcus faecalis] [sr:,streptococcus faecalis]
[ec:1.3.3.1] [de:(dhodehase)] [sp:q47741] [db:swissprot]
36219000_c1_118 2741 6395. . . kd protein
in gpa 5'region (orf4)] [sp:q06425] [db:swissprot]
36220678_c2_122 2743 6397 375 124 237 4.50E-20 [ln:eharpqtou] [ac:z50854]
[gn:orf1] [or:enterococcus hirae] [db:genpept-bct]
[de:**e**.hirae arp[q,r,s,t,u] genes.] [le:<1] [re:150] [di:direct]
36222917_c3_80 2744 6398 1200 399 159 2.50E-08 [ac:p12957:q90756:q90761:q92018:
q99230:q03698] [gn:cald1:cad]
[or:gallus gallus] [sr:,chicken] [de:caldesmon (cdm)] [sp:p12957:q90756:
q90761:q92018:q99230:q03698]. . . in
hydrogenase 1 5'region (fragment)] [sp:q46189] [db:swissprot]
36227013_c3_84 2752 6406 330 109 354 1.80E-32 [ln:charpqtou] [ac:z50854]
[pn:arpr] [gn:arpr] [or:enterococcus hirae]
[db:genpept-bct] [de:**e**.hirae arp[q,r,s,t,u] genes.] [le:327] [re:638]
[di:direct]
36229712_f2_17 2753 6407 480 159 224 1.10E-18 [ac:p44789] [gn:mscl:hi0626]
[or:haemophilus influenzae] [de:large
conductance mechanosensitive channel] [sp:p44789]. . . [gn:mth338]
[or:methanobacterium thermoautotrophicum] [db:pir]
36379693_c2_118 2761 6415 471 156 76 0.1 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[dc:enterococcus faecalis ds16 transposon tn916, (tet(**m**)),
(xis-tn),(int-tn) genes,
orfs 1-24, complete cds, complete sequence.] [nt:orf9] [le:14388]
[re:14741]
[di:complement]
36386505_c2_179 2762 6416 183 60 61 0.37 [ac:s72780] [pn:b1496_f2_65. . .
0.43 [ac:s34499:s34867] [pn:hypothetical protein 177 (psbc 3' region)]
[or:chloroplast
euglena gracilis] [db:pir]
3914033_f3_10 2805 6459 216 71 64 0.41 [ac:s67072] [pn:probable **membrane**
protein yor180c:hypothetical protein o4718]
[gn:ehd2] [or:saccharomyces cerevisiae] [db:pir] [mp:15r]
391575_f3_22 2806 6460 915 304 600 1.50E-58 [ac:g69777] [pn:transcriptional
regulator (arac/xyls famil). . .
DETD . . . phosvitin] [le:<1] [re:
3945462_f2_9 2822 6476 267 88 65 0.16 [ln:efas48c] [ac:y12234] [pn:as-48c1

protein] [gn:as 4061] [in:putative abc
transporter] [or:enterococcus faecalis] [db:genpept-bct] [de:**e**.
faecalis plasmid
dna containing gene cluster involved inproduction and immunity to
peptide
antibiotic as-48.] [le:30
3946930_c2_48 2823 6477 669 222 532. . . [le:21:693:834:1242:1417]
[re:58:785:1181:1365:1575]
[di:directjoin]
3948337_f3_8 2826 6480 867 288 341 4.30E-31 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct] [de:
enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes,
orfs 1-24, complete cds, complete sequence.] [nt:orf20] [le:2861]
[re:3850]
[di:direct]
3948338_f3_4 2827 6481 852 283 563 1.30E-54 [ac:p77791]. . . inheritance
protein (f leading maintenance protein)] [sp:p16077] [db:swissprot]
4010937_c2_19 2843 6497 888 295 1134 4.00E-115 [ac:p42361] [or:streptococcus
gordonii challis] [de:29 kd **membrane** protein
in psaa 5'region orf1)] [sp:p42361] [db:swissprot]
401386_f2_12 2844 6498 207 68 57 0.54 [ln:kpnifh02] [ac:x01007] [or:klebseilla
pneumoniae] [db:genpept-bct]
[de:klebsiella pneumoniae. . . strain=297] [db:genpept-bct]
[de:borrelia burgdorferi 2.9-3
locus, orf-c gene, partial cds, orf-d, rep+, rep-, and lipoprotein (lp)
genes,
complete cds.] [nt:repeat **m**
4023427_c2_60 2848 6502 402 133 53 0.93 [ln:spu20837] [ac:u20837] [gn:emml]
[or:streptococcus pyogenes]
[db:genpept-bct] [de:streptococcus pyogenes **m** type pt5757 (emml) gene,
partial cds.] [le:<1] [re:
4023427_c3_47 2849 6503 2418 805 147 2.80E-09 [ln:llu50902] [ac:u50902]
[pn:ltrc] [gn:ltrc] [or:lactococcus lactis. . . [db:genpept-pln]
[de:l. japonicus mrna for small gtp-binding
protein rabla.] [le:129] [re:734] [di:direct]
4160135_c3_108 2912 6566 216 71 71 0.017 [ac:s67091] [pn:probable **membrane**
protein yor199w:hypothetical protein o4821]
[or:saccharomyces cerevisiae] [db:pir] [mp:15r]
4161578_f3_28 2913 6567 537 178 637 1.80E-62 [ac:p02391] [gn:rplf] [or:bacillus
stearothermophilus] [de:50s ribosomal
. . .
DETD . . . 1-epimerase, (mutarotase)] [sp:p40681] [db:swissprot]
4335963_c1_98 2954 6608 360 119 65 0.073 [ac:o10371] [or:orgyia pseudotsugata
multicapsid polyhedrosis virus] [sr:,
opmnpv] [de:occlusion-derived virus **envelope** protein e18 (odv-e18)]
[sp:o10371]
[db:swissprot]
4336082_c3_67 2955 6609 1092 363 1061 2.20E-107 [ac:f69786] [pn:glycoprotein
endopeptidase homolog ydie] [or:bacillus subtilis]
[db:pir]
4336088_f1_1 2956. . . [pn:abc transporter (atp-binding protein) homolog
yfmr] [gn:yfmr]
[or:bacillus subtilis] [db:pir]
4501075_c3_56 2987 6641 549 182 101 0.0056 [ac:p02977] [gn:emm5:smp5]
[or:streptococcus pyogenes] [de:**m** protein,
serotype 5 precursor] [sp:p02977] [db:swissprot]
4508392_f1_8 2988 6642 1251 416 1069 3.10E-108 [ac:p37535] [gn:yaan]
[or:bacillus subtilis] [de:hypothetical 43.8
kd protein. . . 3021 6675 3084 1027 166 2.70E-11 [ac:s42798]
[pn:fibronectin-binding protein] [or:streptococcus "equisimilis"]
[db:pir]
4744188_f1_2 3022 6676 189 62 56 0.49 [ln:hivser033] [ac:z37845] [pn:**envelope**
protein] [gn:env] [or:human
immunodeficiency virus type 1] [db:genpept-vrl] [de:hiv-1 dna v3 region
(patient

05; sample serum, clone 05).] [nt:v5 region]. . .
DETD . . . 6715 1983 660 193 6.00E-12 [ac:s52348] [pn:hypothetical protein
2] [or:lactobacillus
leichmannii] [db:pir]
4823576_f3_103 3062 6716 798 265 134 6.90E-07 [ac:42926] [pn:hypothetical
membrane spanning protein] [or:
staphylococcus aureus] [db:pir]
4823571_c2_70 3063 6717 393 130 52 0.96 [ac:p20710] [gn:xis] [or:bacteriophage
154a] [de:excisionase]
[sp:p20710] [db:swissprot]
48577_f2_10 3064. . . in
kinc-adec intergenic region (orf4)] [sp:p39760] [db:swissprot]
4886593_f3_44 3114 6768 549 182 517 9.50E-50 [ln:ehcopayz] [ac:z46807] [gn:orf
u] [or:enterococcus hirae] [db:genpept-bct]
[de:**e**. hirae copa, copy and copz genes.] [le:646] [re:1185] [di:direct]
4886643_c3_97 3115 6769 684 227 680 5.10E-67 [ac:s49544] [pn:response
regulator] [cl:response regulator. . . 0.1 [ln:mcu60315] [ac:u60315]
[pn:mc1181] [gn:mc1181] [or:molluscum
contagiosum virus subtype 1] [db:genpept-vrl] [de:molluscum contagiosum
virus subtype 1, complete genome.] [nt:putative virion **membrane** protein;
contains a] [le:139570] [re:139854] [di:complc
4962802_f3_17 3150 6804 201 66 71 0.039 [ac:p07709] [gn:nd6] [or:drosophila
yakuba] [sr:,fruit fly] [ec:1.6.5.3]
[de:nadh-ubiquinone. . .
DETD . . . complete cds, orf70'g
4976587_f3_32 3160 6814 258 85 126 2.60E-08 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct] [de:
enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes,
orfs 1-24, complete cds, complete sequence.] [nt:orf8] [le:15665]
[re:15895]
[di:direct]
4976630_f1_2 3161 6815 228 75 69 0.028 [ac:s24443:s19667]. . . mrna for 3el
protein.] [le:32] [re:418]
[di:direct]
5114042_c3_21 3191 6845 1017 338 295 3.20E-26 [ln:ecmpc7a] [ac:x57583]
[gn:mccf] [fn:immunity] [or:escherichia coli]
[db:genpept-bct] [de:**e**. coli plasmid pmccc7 mcca, b, c, d, **e**, f genes.]
[le:5173] [re:6207] [di:complement]
5114455_c3_74 3192 6846 843 280 688 7.20E-68 [ac:c69693] [pn:ribonuclease h
rnh] [gn:rnh] [or:bacillus subtilis] [db:pir]
5115963_c2_28 3193. . . [ac:p36470] [gn:rps4] [or:rhapis humilis]
[de:chloroplast 30s ribosomal protein s4
(fragment)] [sp:p36470] [db:swissprot]
5117091_c1_56 3196 6850 861 286 544 1.30E-52 [ac:a56641] [pn:probable
membrane transport protein] [or:clostridium
perfringens] [db:pir]
5117186_c1_41 3197 6851 231 76 64 0.13 [ac:p49794] [or:oreochromis mossambicus]
[sr:,mozambique tilapia:
tilapia mossambica] [de:melanin-concentrating hormone. . . [yuf
homolog yufl]
[gn:yufl] [or:bacillus subtilis] [db:pir]
5209718_f3_53 3226 6880 342 113 106 7.90E-05 [ac:p07197] [gn:nefm:nfm] [or:homo
sapiens] [sr:,human] [de:neurofilament
triplet **m** protein (160 kd neurofilament protein) (nf-**m**)] [sp:p07197]
[db:swissprot]
523938_c2_58 3227 6881 312 103 64 0.22 [ac:a69851] [pn:hypothetical protein
yjia] [gn:yjia] [or:bacillus subtilis] [db:pir]
5250311_c1_19 3228 6882 708 235. . .
DETD . . . [or:bacillus subtilis] [de:hypothetical 6.7 kd protein in
spo0a-mmga intergenic region] [sp:p54525] [db:swissprot]
5867300_c1_134 3289 6943 1611 536 145 8.50E-07 [ln:strmry] [ac:m58461] [pn:**m**
protein trans-acting positive regulator] [gn:mry]
[or:streptococcus pyogenes] [sr:streptococcus pyogenes (strain d471)
dna]
[db:genpept-bct] [de:s. pyogenes trans-acting positive regulator of **m**

protein (mly)
gene, complete cds.
5870260_f1_10 3290 6944 258 85 78 0.0032 [ac:p29472] [or:lactobacillus
acidophilus] [de:hypothetical 14.4 kd protein in laf
. . . 6803,] [db:pir]
5959693_c1_61 3303 6957 207 68 80 0.0019 [ln:sac194]
[ac:v01278:j01755:j01756:j01757:j01758:v01279:v01280] [or:
staphylococcus aureus] [db:genpept-bct] [de:s. aureus plasmid pc194
orfs a,b,c,d,**e**, and f.] [nt:reading frame **e**] [le:857] [re:
5978127_c3_28 3304 6958 216 71 73 0.024 [ln:a25773] [ac:a25773] [pn:2s albumin
protein] [or:raphanus sativus
[sr:radish] [db:genpept-pat] [de:r. sativus 2s. . . [ac:q02170]
[gn:ysxa] [or:bacillus subtilis] [de:dna repair protein radc homolog
(orfb)] [sp:q02170] [db:swissprot]
6073412_f3_67 3326 6980 297 98 85 0.079 [ac:s64942:s64943:s69393] [pn:probable
membrane protein ylr106c:hypothetical
protein 12901] [or:saccharomyces cerevisiae] [db:pir] [mp:12r]
6094450_f3_21 3327 6981 534 177 59 0.62 [ln:celt25g12] [ac:u43283]
[gn:t25g12.3] [or:caenorhabditis elegans] [sr:
. . . 636 211 179 5.00E-13 [ac:s52348] [pn:hypothetical protein 2]
[or:lactobacillus leichmannii] [db:pir]
6289058_c3_48 3349 7003 183 60 56 0.21 [ln:tbz56279] [ac:z56279] [pn:integral
membrane protein] [gn:cglg] [or:
thermoanaerobacter brockii] [db:genpept-bct] [de:t. brockii cglf, cglg,
xgls
and cglt genes.] [le:1135] [re:1977] [di:direct]
6299062_c1_99 3350 7004 243. . . [ac:p17161:p11377] [or:klebsiella
pneumoniae] [de:probable sigma (54)
modulation protein (orf95)] [sp:p17161:p11377] [db:swissprot]
6301575_c2_74 3351 7005 270 89 56 0.49 [ln:rnu75406] [ac:u75406] [pn:lysosomal
membrane glycoprotein] [gn:lamp-1]
[or:rattus norvegicus] [sr:norway rat] [db:genpept-rod] [de:rattus
norvegicus
lysosomal **membrane** glycoprotein (lamp-1) mrna, partial cds.] [le:<1]
[re:
6301712_c3_54 3352 7006 627 208 687 9.20E-68 [ac:b69878] [pn:guanylate kinase
homolog ylod] [gn:ylod] [or:bacillus. . . 570 189 148 1.20E-10
[ln:mtcy01b2] [ac:z95554] [pn:unknown] [gn:mtcy01b2.16c]
[or:mycobacterium
tuberculosis] [db:genpept-bct] [de:mycobacterium tuberculosis cosmid
scy01b2.] [nt:mtcy01b2.16c. len: 195. function: unknown **membrane**]
[le:17189]
[re:17776] [di:complement]
6438177_f1_5 3369 7023 423 140 76 0.43 [ln:celb0496] [ac:u58749] [gn:b0496.4]
[or:caenorhabditis elegans] [sr:
caenorhabditis elegans strain=bristol n2] [db:genpept-inv]. . .
DETD . . . 203 125 8.40E-07 [ln:mtcy01b2] [ac:z95554] [pn:unknown]
[gn:mtcy01b2.16c] [or:
mycobacterium tuberculosis] [db:genpept-bct] [de:mycobacterium
tuberculosis cosmid scy01b2.] [nt:mtcy01b2.16c. len: 195. function:
unknown
membrane] [le:17189] [re:17776] [di:complement]
6652_f2_78 3389 7043 186 61 69 0.032 [ac:p22450] [or:haloarcula marismortui]
[sp:,halobacterium marismortui] [de:
50s ribosomal protein 114 (hmal14) (h127)] [sp:p22450] [db:swissprot]
6656303_f3_99 3390 7044 393 130 64 0.12 [ln:mc311] [ac:z33228] [pn:similar to
7-alpha-hydroxysteroid dh] [or:
mycoplasma capricolum] [db:genpept-bct] [de:**m**. capricolum dna for
contig mc311.] [nt:orf identified by homology to swissprot entry]
[le:<1]
[re:253] [di:direct]
6657660_f3_84 3391 7045 1191 396 468. . . 80 0.087 [ac:p43978] [gn:hi0284]
[or:haemophilus influenzae] [de:hypothetical protein
hi0284] [sp:p43978] [db:swissprot]
6766877_c1_46 3416 7070 1053 350 468 1.50E-44 [ln:llpflmg13] [ac:aj000325]
[pn:putative **membrane** protein] [gn:orfa] [or:

lactococcus lactis] [db:genpept-bct] [de:lactococcus lactis pil gene
(strain
mg 1363).] [le:270] [re:1187] [di:direct]
6767133_f3_52 3417 7071 312 103. . . faecalis plasmid pad1 gene.]
[nt:structural gene for ultraviolet resistance]
[le:1284] [re:2612] [di:direct]
6929687_f2_8 3445 7099 648 215 141 6.70E-10 [ln:bamalamya] [ac:z22520]
[pn:**membrane** protein] [or:bacillus
acidopullulyticus] [db:genpept-bct] [de:b. acidopullulyticus encoding
maltogenic
amylase.] [nt:putative cds; orf3 highly hydrophobic aa sequence;]
[le:3540]
[re:4091] [di:complement]
6929688_f1_2 3446. . .
DETD . . . for cardiac tropomyosin.]
[sp:q91489] [le:1] [re:
7120692_c3_10 3478 7132 183 60 68 0.22 [ln:efplscplg] [ac:x96976]
[pn:transposase] [gn:tnp1062] [or:
enterococcus faecalis] [db:genpept-bct] [de:**e**. faecalis plasmid dna
sep1 gene,
4068bp.] [le:2496] [re:3455] [di:complement]
7160338_f3_4 3479 7133 381 126 76 0.018 [ac:p44301] [gn:hi1737] [or:haemophilus
influenzae] [de:hypothetical
. . . protein (acp)] [sp:p20804:p73284] [db:swissprot]
7244437_c2_80 3490 7144 1155 384 560 2.70E-54 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tct(**m**)), (xis-tn),
(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf14] [le:9816]
[re:10817] [di:direct]
7267192_c2_110 3491 7145 354 117 147 1.50E-10 [ac:c41868]. . . subtilis]
[db:pir]
781280_c1_45 3501 7155 207 68 68 0.4 [ln:efas48c] [ac:y12234] [pn:as-48b
protein] [gn:as-48b] [fn:as-48 maturation and
biosynthesis] [or:enterococcus faecalis] [db:genpept-bct] [de:**e**.
faecalis
plasmid dna containing gene cluster involved inproduction and immunity
to
peptide antibiotic as-48.]
7812_c2_84 3502 7156 1551 516 183 3.60E-10. . . 65 0.86 [ac:q27783]
[or:trypanosoma brucei brucei] [ec:1.5.1.3:2.1.1.45] [de:(ec
2.1.1.45) (dhfr-ts)] [sp:q27783] [db:swissprot]
892283_c1_50 3553 7207 219 72 72 0.15 [ac:s65238] [pn:probable **membrane**
protein yp1219w:hypothetical protein p1745]
[gn:pc18] [or:saccharomyces cerevisiae] [db:pir] [mp:161]
892327_c3_95 3554 7208 444 147 78 0.48 [ln:ratlin3a] [ac:m13100] [pn:unknown
protein] [or:rattus. . . [ac:p54154] [gn:yppp] [or:bacillus subtilis]
[de:reductase)] [sp:p54154]
[db:swissprot]
9018758_c2_31 3561 7215 693 230 535 1.20E-51 [ac:p53579] [gn:slr0918]
[or:synechocystis sp] [sr:pcc 6803,] [ec:3.4.11.18]
[de:**m**)] [sp:p53579] [db:swissprot]
912_c2_15 3562 7216 945 314 114 0.00091 [ac:q09316] [gn:f25b5.5]
[or:caenorhabditis elegans] [de:hypothetical 61.3 kd
protein f25b5.5 in chromosome iii]. . . sp. brain] [db:genpept-rod]
[de:machr=mm4
muscarinic acetylcholine receptor [mice, brain, mrna partial, 362 nt].]
[nt:this sequence comes from FIG. 2b; mm4 **m**
94092_c2_21 3565 7219 813 270 667 1.20E-65 [ac:b69477] [pn:abc transporter,
atp-binding protein homolog] [or:archaeoglobus
fulgidus] [db:pir]
9455_f1_1 3566 7220 792 263 174. . . [pn:hypothetical protein yvqf]
[gn:yvqf] [or:bacillus subtilis]
[db:pir]
964203_f3_8 3574 7228 339 112 529 5.10E-51 [ln:efentaorf] [ac:x94181] [gn:orf2]
[or:enterococcus faecium] [db:

genpept bct] [de:e. faecium enta and orf2 genes.] [le:507] [le:610]
[di:direct]
964687_f1_3 3575 7229 594 197 60 0.59 [ln:hivlu14546] [ac:u14546]
[pn:**envelope** glycoprotein] [gn:env] [or:human
immunodeficiency virus type 1] [db:genpept-vrl] [de:human
immunodeficiency
virus type 1 clone mp49a **envelope** glycoprotein (env) gene, partial cds.]
[le:<1] [re:
970287_c3_83 3576 7230 501 166 159 8.30E-12 [ac:p37187:p76413] [gn:gata]
[or:escherichia coli] [ec:2.7.1.69]
[de:(ec 2.7.1.69)]. . .
DETD . . . (upd-n-acetylmuramyl-tripeptide synthetase)] [sp:q03523]
[db:swissprot]
9797032_c2_14 3595 7249 483 160 204 2.40E-16 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
[de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn) genes,
orfs 1-24, complete cds, complete sequence.] [nt:orf14] [le:9816]
[re:10817]
[di:direct]
979713_c2_21 3596 7250 1785 594 643 4.20E-63 [ac:q11018]. . . [gn:uvra]
[or:bacillus
subtilis] [db:pir]
984536_c2_154 3612 7266 1209 402 1160 7.00E-118 [ln:efu09422] [ac:u09422]
[or:enterococcus faecalis] [db:genpept-bct]
de:enterococcus faecalis ds16 transposon tn916, (tet(**m**)), (xis-tn),
(int-tn)
genes, orfs 1-24, complete cds, complete sequence.] [nt:orf20] [le:2861]
[re:3850] [di:direct]
9847200_c1_28 3613 7267 1374 457 1382 2.10E-141 [ac:s41386]. . . potassium
transporter
homolog gene, partial cds.] [le:898
987562_c2_53 3625 7279 195 64 47 0.13 [ln:efas48c] [ac:y12234] [pn:hypothetical
protein] [or:enterococcus faecalis]
[db:genpept-bct] [de:**e**.faecalis plasmid dna containing gene cluster
involved
inproduction and immunity to peptide antibiotic as-48.] [nt:orf6]
[le:4556]
[re:5065] [di:direct]
9882952_f2_19 3626 7280 1053. . . 532 635 3.00E-62 [ln:celk11g12]
[ac:u23525] [gn:k11g12.4] [or:caenorhabditis elegans] [sr:
caenorhabditis elegans strain=bristol n2] [db:genpept-inv]
[de:caenorhabditis
elegans cosmid k11g12.] [nt:similar to **m**. musculus transport system
membrane]
[le:17077:17447:17654] [re
995328_c2_5 3646 7300 423 141 152 4.60E-11 [ac:g69980] [pn:hypothetical protein
yrvd] [gn:yrvd] [or:bacillus subtilis]
[db:pir]
995437_c2_251 3647 7301 813 270. . .
1. An isolated nucleic acid consisting of a nucleotide sequence encoding
an **E**. faecium polypeptide selected from the group consisting of SEQ ID
NO: 3857, SEQ ID NO: 4234, SEQ ID NO: 4304,. . .
9. An isolated nucleic acid consisting of a nucleotide sequence encoding
an **E**. faecium polypeptide wherein said isolated nucleic acid consists
of at least 40 sequential nucleotides selected from the group consisting
of. . .
. . . ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said
isolated nucleic acid encodes an **E**. faecium polypeptide.

. . . ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said
isolated nucleic acid encodes an **E**. faecium polypeptide selected from
the group consisting of SEQ ID NO: 3857, SEQ ID NO: 4234, SEQ ID NO:
4304,. . .
. . . ID NO: 2502, SEQ ID NO: 2666, and SEQ ID NO: 3570 and wherein said
isolated nucleic acid encodes an **E**. faecium polypeptide selected from

the group consisting of SEQ ID NO: 3057, SEQ ID NO: 4254, SEQ ID NO: 4304,. . .

28. An isolated nucleic acid consisting of a nucleotide sequence encoding an **E**. faecium polypeptide wherein said isolated nucleic acid consists of at least 30 sequential nucleotides selected from the group consisting of. . .

31. An isolated nucleic acid consisting of a nucleic acid encoding an **E**. faecium polypeptide wherein said isolated nucleic acid consists of at least 20 sequential nucleotides selected from the group consisting of. . .

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=> d his

     (FILE 'HOME' ENTERED AT 15:58:13 ON 30 APR 2004)

     FILE 'USPATFULL' ENTERED AT 15:59:07 ON 30 APR 2004
               E CHANG GWONG JEN/IN
L1             1 S E4

     FILE 'WPIDS' ENTERED AT 16:00:09 ON 30 APR 2004
               E CHANG G J/IN
L2           106 S E3
L3             2 S L2 AND FLAVIVIR?

     FILE 'MEDLINE' ENTERED AT 16:01:13 ON 30 APR 2004
               E CHANG G J/AU
L4            49 S E3
L5            29 S L4 AND (FLAVIVIR? OR YELLOW OR DENGUE OR ENCEPHALITIS)
L6             9 S L5 AND (PRM OR E OR PRM/M OR PRM/E)

     FILE 'USPATFULL' ENTERED AT 16:06:37 ON 30 APR 2004
L7          3470 S (FLAVIVIR? OR YELLOW FEVER VIR? OR DENGUE OR ENCEPHALITIS VIR
L8            79 S L7 AND (PRM OR PREMEMBRANE)
L9            79 S L8 AND (E OR ENVELOPE)
L10           79 S L9 AND (M OR MEMBRANE)
L11           43 S L10 AND (SIGNAL SEQUENCE)
L12            5 S L11 AND KOZAK
L13           38 S L11 NOT L12
L14           15 S L13 AND AY<1999

=> d l14,cbib,ab,clm,kwic,2-15
```

prM gene

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L14  ANSWER 2 OF 15  USPATFULL on STN
2002:246731 Dengue nucleic acid vaccines that induce neutralizing antibodies.
    Kochel, Tadeusz J., Frederick, MD, United States
    Porter, Kevin R., Gaithersburg, MD, United States
    Raviprakash, Kanakatte, Silver Spring, MD, United States
    Hoffman, Stephen L., Gaithersburg, MD, United States
    Hayes, Curtis G., Frederick, MD, United States
    The United States of America as represented by the Secretary of the Navy,
    Washington, DC, United States (U.S. government)
    US 6455509 B1 20020924
    APPLICATION: US 1997-869423  19970604 (8)                    <--
    PRIORITY: US 1996-17839P  19960604 (60)
    DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB     A vaccine for promoting an immune response in a mammalian subject
       includes a eucaryotic plasmid expression vector which include at least
       part of the envelope gene and optionally, the PreM gene of dengue
       virus. In order to minimize immune enhancement, vaccines of up to the
       four serotypes of dengue are combined in a single vaccine. The vaccine
       in a suitable pharmaceutical carrier constitutes a pharmaceutical
       composition which is injected into the subject.
CLM    What is claimed is:
       1. A pharmaceutical composition capable of inducing an immune response
```

in a mammalian subject, comprising an immunogenic amount of a eukaryotic plasmid expression vector in pharmaceutically acceptable form, wherein said plasmid expression vector is functional in mammalian subjects and includes preM and at least 92% of the **envelope** gene of a **dengue** W virus, where W is a number selected from the group consisting of 1, 2, 3 and 4.

2. The pharmaceutical composition of claim 1 further comprising a second plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** X virus, where X is a number different from W and is selected from a the group consisting of 1, 2, 3 and 4.

3. The pharmaceutical composition of claim 2 further comprising a third plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** Y virus, where Y is a number different from W and from X and is selected from the group consisting of 1, 2, 3 and 4.

4. The pharmaceutical composition of claim 3 further comprising a fourth plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** Z virus, where Z is a number different from W, from X, and from Y, and is selected from the group consisting of 1, 2, 3 and 4.

5. The pharmaceutical composition of claim 1, further comprising a suitable pharmaceutical carrier.

6. The pharmaceutical composition of claim 5, which is in injectable form.

7. The pharmaceutical composition of claim 1, which is a vaccine capable of inducing a protective immune response in said mammalian subject, comprising an immunoprotective amount of said plasmid expression vector.

8. The vaccine of claim 7, which comprises a suitable pharmaceutical carrier, and is in injectable form.

9. A method of inducing an immune response in a mammalian subject, comprising the step of injecting the composition of claim 6.

10. A method of inducing a protective immune response in a mammalian subjects comprising the step of injecting the vaccine of claim 8.

11. The pharmaceutical composition of claim 2, further comprising a suitable pharmaceutical carrier.

12. The pharmaceutical composition of claim 11, which is in injectable form.

13. The pharmaceutical composition of claim 2, which is a vaccine capable of inducing a protective immune response in said mammalian subject, comprising an immunoprotective amount of said plasmid expression vector.

14. The vaccine of claim 13, which comprises a suitable pharmaceutical carrier, and is in injectable form.

15. A method of inducing an immune response in a mammalian subject, comprising the step of injecting the composition of claim 12.

16. A method of inducing a protective immune response in a mammalian subject comprising the step of injecting the vaccine of claim 14.

17. The pharmaceutical composition of claim 3, further comprising a suitable pharmaceutical carrier.

18. The pharmaceutical composition of claim 17, which is in injectable form.

19. The pharmaceutical composition of claim 3, which is a vaccine capable of inducing a protective immune response in said mammalian subject, comprising an immunoprotective amount of said plasmid expression vector.

20. The vaccine of claim 19, which comprises a suitable pharmaceutical carrier, and is in injectable form.

21. A method of inducing an immune response in a mammalian subject, comprising the step of injecting the composition of claim 18.

22. A method of inducing a protective immune response in a mammalian subject, comprising the step of injecting the vaccine of claim 20.

23. The pharmaceutical composition of claim 4, further comprising a suitable pharmaceutical carrier.

24. The pharmaceutical composition of claim 23, which is in injectable form.

25. The pharmaceutical composition of claim 4, which is a vaccine capable of inducing a protective immune response in said mammalian subject, comprising an immunoprotective amount of said plasmid expression vector.

26. The vaccine of claim 25, which comprises a suitable pharmaceutical carrier, and is in injectable form.

27. A method of inducing an immune response in a mammalian subject, comprising the step of injecting the composition of claim 24.

28. A method of inducing a protective immune response in a mammalian subject, comprising the step of injecting the vaccine of claim 26.

TI **Dengue** nucleic acid vaccines that induce neutralizing antibodies

AI US 1997-869423 19970604 (8) <--

AB . . . an immune response in a mammalian subject includes a eucaryotic plasmid expression vector which include at least part of the **envelope** gene and optionally, the PreM gene of **dengue** virus. In order to minimize immune enhancement, vaccines of up to the four serotypes of **dengue** are combined in a single vaccine. The vaccine in a suitable pharmaceutical carrier constitutes a pharmaceutical composition which is injected. . .

PARN This application is related to the Provisional Application for Patent entitled **Dengue** Nucleic Acid Vaccines That Induce Neutralizing Antibodies filed Jun. 4, 1996 by the inventors Tadeusz Kochel, Kevin R. Porter, Stephen. . .

SUMM This invention relates to nucleic acid vaccines and more specifically to **Dengue** nucleic acid vaccines.

SUMM **Dengue** (Den) viruses belong to the **flavivirus** genus of the family **Flaviviridae** and are of four serotypes, Den 1-4. **Dengue** viruses are positive strand RNA viruses which code for ten genes. The genes are translated as a polyprotein which is cleaved by host and viral proteinases. The virus **envelope** (**E**) protein is the major antigen against which neutralizing antibodies are directed. These antibodies have been shown to be capable of protecting against **dengue** virus infection[1]. The **membrane** protein also appears on the virion surface and is required for the proper processing of **E**.

SUMM **Dengue** viruses are transmitted primarily by the mosquito, Aedes aegypti, and are a major cause of morbidity and mortality throughout tropical and subtropical regions worldwide[2]. It is estimated that there are over 100 million cases, annually, of **dengue** fever[3]. Human **dengue** illnesses range from an acute undifferentiated fever to **dengue** hemorrhagic fever (DHF) and **dengue** shock syndrome (DSS). A primary infection usually causes **dengue** fever. The illness is

generally mild and the person apparently acquires a life long immunity against the serotype of **dengue** virus causing the infection. However, if a person acquires a second **dengue** infection with a different serotype, the illness may be more severe and lead to hemorrhagic fever or shock syndrome, with. . . is caused by an immune enhancement phenomenon[4]. Immune enhancement begins when non-neutralizing antibodies, generated from the earlier infection with one **dengue** serotype, bind to but do not neutralize the virus causing the secondary infection. The Fc portion of the antibody in. . .

SUMM At present there is no approved vaccine for **dengue** viruses. The most effective **dengue** vaccine would elicit sustained protective levels of neutralizing antibodies against all four serotypes so as to avoid the possibility of immune enhancement in a vaccinated individual who might become secondarily infected with a different epidemic or endemic **dengue** of a different serotype.

SUMM Work in mice and primates with inactivated whole virus and at recombinant protein **dengue** vaccines has generally been disappointing because of the lack of sustained protective neutralizing antibodies induced. Vaccination with live attenuated **dengue** virus vaccines is another promising approach, but difficulties still remain in developing a product that is immunogenic and does not cause **dengue** fever-like side effects. DNA vaccines for **dengue** will offer substantial advantages over these other approaches in that sustained immunity can be achieved without the risk of **dengue** fever-like side effects or immune-enhancement.

SUMM It is an object of this invention to protect a subject or community against infection by **dengue** virus.

SUMM It is another object of this invention to provide protection against infection by **dengue** virus by using a nucleic acid vaccine.

SUMM It is an object of this invention to provide protection against more than one **dengue** virus serotype.

SUMM These and additional objects of the invention are accomplished by taking the **envelope** (**E**) and optionally, the **membrane** (PreM) genes of **dengue** virus, serotypes 1, 2, 3, and 4 and cloning them into eukaryotic plasmid expression vectors. The resultant plasmid DNAs (**dengue** DNA vaccines) are injected into a mammalian subject where in vivo the **E** proteins are expressed and subsequently recognized and processed by immune cells. This results in the generation of humoral and cellular immunity that is protective against **dengue** virus infections of each of the four serotypes.

SUMM . . . potential as Den virus vaccines. The Den genes which we have incorporated into our DNA vaccines include: 80-100% of the **E** gene; either alone or expressed with the PreM gene. The choice of these genes systems from publications which demonstrate that: **E** contains the virus' major antigenic determinants[11]; not all of **E** is required to obtain a protective immune response[12]; and PreM affects the conformation of the produced **E** protein[13]. As Den virus DNA vaccines, some combinations of the above-listed genes yield better immune responses than others.

DRWD FIG. 5 is a Kaplan Meier Survival plot summarizing the results of a mouse challenge experiment using the **dengue** type 2 DNA vaccine.

DRWD FIG. 6 is a graph showing the immunogenicity of a Den 1 DNA vaccine containing 80% **E**.

DRWD FIG. 7 is a graph illustrating the immunogenicity of DNA vaccines constructed against **dengue** serotype 1 and containing the PreM gene and different amounts of the **E** gene.

DRWD FIG. 8 is a graph illustrating the immunogenicity of DNA vaccines constructed against **dengue** serotype 2 and against **dengue** serotype 3.

DETD By this invention the newly developed nucleic acid vaccination approach is adapted to provide a **dengue** virus vaccine, also called a naked DNA **dengue** vaccine. Eukaryotic plasmid expression vectors containing the PreM and at least part of the **E** gene of a virus selected from the group consisting of **dengue**-1 virus, **dengue**-2 virus, **dengue**-3 virus and **dengue**-4 virus are formed. The constructs are then injected into a mammalian subject; either individually or in combinations including up to. . . cellular immunity are long lasting. The invention applies

nucleic acid vaccine technology to develop a DNA plasmid construct that elicits anti-**dengue** neutralizing antibodies in a mouse model which leads to and is adaptable to later application to other mammals, including non-human. . .

DETD In general, the naked DNA **dengue** vaccine of the present invention includes the **dengue** preM gene, and at least part of the **dengue E** gene, for **dengue** type 1, 2, 3 or 4, or some combination of types 1, 2, 3 and 4. The published sequences for the preM and **E** genes of **dengue** serotype 1, West Pacific strain, serotype 2, New Guinea C strain, serotype 3, H87 strain, and serotype 4, H81 strain,. . . The vaccine preferably includes at least about 80% and most preferably, about 100%, about 92% or about 80% of the **dengue E** gene. Alternatively, the vaccine of the present invention preferably includes about 100%, about 92% or about 80% of the **dengue E** gene and need not include any of the preM gene. Silent base substitutions may be made to the above-described DNA without altering the effectiveness or scope of the present invention. The DNA **dengue** vaccine of the present invention does not include any nucleotides or sequences susceptible to expression in a mammalian cell other. . .

DETD . . . The promoter/enhancer works with RNA polymerase to initiate transcription of mRNA and the cloning site is for insertion of the **dengue** genes of interest. The expression vector preferably also includes an intron, which is not expressed in the protein produced by. . .

DETD This invention shows that the nucleic acid vaccination technique can be used to raise a protective anti-**dengue** virus immune response in mice. In the first series of experiments, two different plasmid expression vectors, pkCMVintPolyli and pVR1012 were. . . Bovine Growth Hormone termination sequence. p1012 does not include the SV40 origin of replication. The Den-2 genes PreM and 92% **E** were cloned into pkCMVintPolyli and p1012 (see "Examples" below for cloning details); the resultant constructs are pD2ME and p1012D2ME, respectively.. . .

DETD To determine if pD2ME and p1012D2ME properly express the **E** gene in vitro, Immune Fluorescence Assays (IFA) and Radio-labeled Immune Precipitation Analysis (RIPA) were performed on transiently transfected cells. Details. . .

DETD . . . and spotted onto slides. The slides were then reacted with either Den-2 hyperimmune ascitic fluid (HIAF) or conformation specific anti-Den **envelope** antibodies 4G2 and 3H5. Fluorescein conjugated # secondary antibody was used to visualize the primary antibody-antigen interaction by fluorescence.. . .

DETD Table I shows that cells transfected with either pD2ME or p1012D2ME express the truncated **E** protein of Den-2. The ability of Den-2 Hyper-Immune Ascitic Fluid (HIAF) to recognize the truncated **E** protein in the IFA demonstrates the expression of the truncated **E** protein. No truncated **E** protein is expressed in cells transfected with vector only (i.**e**. pkCMVintPolyli or p1012). The proper expression of the truncated **E** gene, and retention of conformational epitopes, in vitro, is demonstrated by the ability of two, Den-2, conformation-dependent monoclonal antibodies, 4G2. . .

DETD . . . seen in RIPA of identically transfected cells. Den-2 HIAF, 3H5 and 4G2 each immune precipitated greater amounts of the truncated **E** protein from cell lysates of p1012D2ME transfected cells than from pD2ME-transfected cells. RIPA also detected truncated **E** protein in the media of transfected cells demonstrating that the truncated **E** protein is secreted (data not shown).

DETD . . . course of antibody production is shown. The three week old mice that were intradermally inoculated with pD2ME or p1012D2ME produced **dengue** antibodies, as manifested by ELISA results. Mice inoculated with pkCMVintPolyli or pVR10122 did not produce any Den antibodies. The mean. . .

DETD . . . protection from a lethal challenge of virus unequivocally demonstrates the efficacy of the vaccine. In the murine system, the standard **dengue** virus challenge is conducted with six week old mice since older mice are less susceptible to the virus[16]. Three week. . .

TD . . . Groups of 5 mice were immunized ID with either 200 µg, 50 µg, 12.5 µg or 3.1 µg of the **dengue** 2 DNA vaccine p1012D2ME92. Additional groups of mice were co-immunized with the same amounts of vaccine together with 100 µg. . .

TD . . . 10 and 25 and serum samples obtained on days 25 and 38. At day 25 (after a single boost) the **dengue** antibody response, as measured by mean OD value, in mice that received 3.1 µg and 12.5 µg of p1012D2ME was. . . mean OD value compared to the mice that received the same dose along with pUC (FIG. 3). The detection of **dengue** antibody at day 25 in the 200 µg group contrasts earlier findings where **dengue** antibodies were not seen until approximately two months post-priming. The reason for this difference is not clear, but may be. . .

TD . . . seen between the groups that were immunized with 3.1 µg ($p<0.05$), indicating that pUC significantly enhanced the immunogenicity of the **dengue** DNA vaccine at the lowest dosage.

TD . . . immunized with vaccine alone. These results indicate that ISS contained in pUC significantly enhances the neutralizing antibody response of the **dengue** DNA vaccine and that 100 µg of pUC 19 produced the greatest immunostimulatory effect.

TD . . . describes Den 2 DNA vaccines. To demonstrate the feasibility of constructing a DNA vaccine against another serotype, 80% of the **E** gene of Den type 1 virus was cloned into the plasmid vector pkCMVintPolyli; the resultant plasmid is pD1E80 (details of cloning, in vitro, and in vivo experiments are described further below under "Examples"). Proper expression of 80% **E** off pD1E80, in vitro, was confirmed by IFA and RIPA. In vitro, 80% **E** was made in cells and secreted from the cells into the cell culture media (data not shown).

TD The Den-1 80% **E** gene expressed from pkCMVintPolyli yielded a significant immune response in mice. Since in the **dengue** 2 experiments genes cloned into the other vector p1012 yielded greater antibody responses than the same genes cloned into pkMVintPolyli, Den 1 genes were cloned into p1012. To evaluate how much of the **E** gene to include to produce the optimum antibody response, DNA vaccine candidates p1012D1E80, p1012D1ME92, p1012D1ME80 and p1012D1ME100 were prepared. p1012D1E80 has 80% of Den 1 **E** cloned into p1012; p1012D1ME92 has the PreM and 92% of Den 1 **E** cloned into p1012; p1012D1ME80 has the PreM and 80% of Den 1 **E** cloned into p1012 and p1012D1ME100 has the PreM and 100% of Den 1 **E** cloned into p1012. All constructs were confirmed to express their **E** gene in vitro by IFA and RIPA (data not shown). To determine if the constructs could induce an immune response. . . Mice immunized with p1012D180 or p1012D1ME100 did produce Den 1 antibodies. The DNA vaccine candidate containing the PreM and 100% **E** elicited the best immune response.

ETD The results presented in this application demonstrate the ability of **dengue** DNA vaccines for **dengue** types 1 and 2 to elicit **dengue** antibody responses in mice. The **dengue** type 2 DNA vaccine was shown to provide significant protection against lethal **dengue** virus challenge. The neutralizing antibodies produced by the **dengue** DNA vaccine were shown to be long-lasting. Referring now to FIG. 8, similar DNA vaccine candidates that express the PreM and 100%**E** genes from Den virus serotype 2 and 3 produce **dengue** antibodies in mice as measured by ELISA. Mice have been inoculated with similar constructs for **dengue** 4 and it is expected that these constructs will have similar effects in producing **dengue** antibodies. A tetravalent **dengue** DNA vaccine that provides protection against all four serotypes can be prepared by combining the four different DNA vaccines to. . . individual vaccines or the individual genes. It is expected that such a combined DNA vaccine would provide life-long protection against **dengue** virus infection without the risk of vaccine induced **dengue** fever-like side effects or the risk of making vaccinated persons vulnerable to the most severe clinical forms of the disease,. . .

DETD The plasmid vectors pkCMVintPolyli and pVR1012, used to construct the **dengue** DNA vaccines were kindly provided by Vical, Inc. PkCMVintPolyli is an expression vector which contains the CMV enhancer, CMV intron,. .

. multiple cloning site, and the Bovine Growth Hormone termination sequence. p1012 does not include the SV40 origin of replication. The **dengue** serotype 2 PreM/**E** genes were derived from a plasmid pkT2.4[18]. This plasmid contains the capsid (C), pre-**membrane** and **envelope** genes of **dengue**-2, New Guinea C strain. The **dengue** sequences spanning bases 1-2249 (including C, PreM and 92% of **E**) were retrieved from this plasmid by EcoR1 restriction enzyme digestion. The EcoR1 restriction fragment was polished with PFU polymerase (Statagene. . . digested with Pst I, gel purified and re-ligated. The Pst I digestion removes sequences from the multiple cloning site to **dengue**-2 base 327. The resultant plasmid is called pD2ME and contains PreM and 92% **E** (bases 327-2249). pD2ME was sequenced on an ABI 377 automated DNA sequencer using Den-2 primers: P2, P4, P5 and P9. . . to the published Den-2 New Guinea C sequence[19], pD2ME contains two point mutations and sequences at the 3' end of **E** which code for seven non-Den-2 amino acids. The first point mutation is a G to A at base 532 and. . . first mutation changes amino acid 68 of PreM from arginine to lysine and the second changes amino acid 454 of **E** from threonine to asparagine. Translation of pD2ME transcripts is expected to begin at codon 103 of Den-2 (base 330), which is the **signal sequence** for PreM, and proceed through amino acid 462 of **E**. Following amino acid 462 are the seven non-Den-2 amino acids (methionine, glutamic acid, leucine, serine, arginine, proline and leucine) and. . .

DETD To construct p1012D2ME, the Den-2 PreM/92% **E** fragment in pD2ME was removed by Pst I/Bgl II digestion and ligated into the multiple cloning site (Pst I/Bgl II. . .

DETD . . . ATT ACT TCT TGA ACC AGC TTA GTT TCA

d: 5'-AGT GGA TCC TCA TTA CTT CAT GGT CCA AGA AAC ACC

e: 5'-AGT GGA TCC TCA TTA CGC CTG AAC CAT GAC TCC TAG

Genome Coordinates Protein encoded Primer pairs Designation

863-2089 80% **E** b&c E80

369-2089 preM+80% **E** a&c ME80

369-2275 preM+92% **E** a&d ME92

369-2392 preM+100% **E** a&**e** ME100

DETD To make the Den-4 construct p1012D4ME100, the PreM and the entire **E** gene of Den-4 (Den-4 strain H421) was made by RT/PCR and cloned into pVR1012. Den-4 virus RNA was purified using. . .

DETD To make the Den-3 construct p1012D3ME100, the PreM and the entire **E** gene of Den-3 (Den-3 strain H87) was made by RT/PCR and cloned into pVR1012. Den-3 virus RNA was purified using. . .

DETD Plasmids were transformed into **E**. coli DH5 cells, grown in the presence of kanamycin and purified by alkaline lysis[20]. Following alkaline lysis, the plasmids were. . .

DETD . . . in 2 ml methionine deficient media for 12 hours. The media and the cells were then subject to immunoprecipitation with **dengue**-specific antisera. Prior to immunoprecipitation the media was removed and the cells were scraped off the plates and lysed in 1 ml RIPA buffer (0.05 **M** Tris-HCl (pH 7.5), 0.15 **M** NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS). For the immunoprecipitations, media or 300 µl cell lysate was. . . at 4° C. for 1 hour. The precipitates were then collected, washed twice with PBS, resuspended in Laemmli buffer (0.015 **M** Tris-HCl (pH 6.8) 0.1 **M** DTT, 2% SDS, 0.1% Bromophenol Blue, 10% glycerol), boiled for 10 minutes followed by 10% SDS-PAGE[22]. The gel was then. . .

DETD . . . were obtained by the periorbital route. Blood samples were obtained approximately two weeks after the last boost were analyzed for **dengue**-specific antibodies using ELISA[24] and the plaque reduction neutralization test[25]. Sera from these samples were stored at -70° C. until used.. . .

DETD Use of the **dengue** DNA vaccines to immunize humans.

DETD In animals DNA vaccines against **dengue** have been shown to elicit neutralizing antibodies that result in significant protection against lethal live-virus challenge. Human immunization, given either IM or ID, with **dengue** DNA vaccines should provide protection against infection

by all four **dengue** serotypes.
DETD . . . X. & Roehrig, J. T. Immunochemistry of Viruses Vol. 2 The basis for Serodiagnosis and Vaccines (eds. van Regen Mortel, **M**. H. V. & Neurgth, A. R.) 289-305 (Elsevier Science (Biomedical Division), Amsterdam, 1990).
DETD 2. Halstead, S. B., 1984. Selective primary health care: Strategies for control of disease in the developing world. XI. **Dengue**. Rev. Infect. Dis., 6:251-264.
DETD 3. Halstead, S. B., Pathogenesis of **Dengue**: Challenges to Molecular Biology. Science 1988;239:476-481.
DETD 4. Kinks, S., Antibody-enhanced Infection of Monocytes as the Pathogenic Mechanism for Severe **Dengue** Illness. Aides Research and Human Retroviruses 1990; 6:993-998.
DETD 6. Ulmer, J. B., Donnelly, J. J., Parker, S. **E**., Rhodes, G. H., Felgner, P. l., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. **M**., Friuedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L., Liu, **M**. A. Heterologous Protection Against Influenza by Injection of DNA Encoding a Viral Protein. Science 259, 1745-1749.
DETD 7. Wang, B., Ugen, K. **E**., Srikantan, V., Agadjanyan, **M**. J., Dang, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, W. V., Weiner, D. B. Gene Inoculation Generates Immune. . .
DETD 8. Major, **M**. **E**., Vitvitski, L., Mink, **M**. A., Schleef, **M**., Whalen, R. G., Trepo, C., Inchauspe, G. DNA-Based Immunization with Chimeric Vectors for the Induction of Immune Responses against the. . .
DETD 9. Sedegah, **M**., Hedstrom, R., Hobart, P., Hoffman, S. Protection Against Malaria by Immunization with Plasmid DNA Encoding Circumsporozoite Protein. Proc. Natl. Acad.. . .
DETD 11. Schlesinger, W. 1977. **Dengue** Viruses. Virol Monogr 16:1 132.
DETD 12. Men, R., Bray, **M**., Lai, C. J. Carboxy-terminally Truncated **Dengue** Virus **Envelope** Glycoproteins Expressed on the Cell Surface and Secreted Extracellularly Exhibit Increased Immunogenicity in Mice. J. Virol. 65:1400-1407.
DETD 13. Konishi, **E**., Mason, P. W. Proper Maturation of the Japanese **Encephalitis Virus Envelope** Glycoprotein Requires Cosynthesis with the **Premembrane** Protein. Journal of Virology, Mar. 1993, p. 1672-1675.
DETD 14. Delenda, C., Frenkiel, **M**., Deubel, V. Protective Efficacy in Mice of a Secreted form of Recombinant **Dengue**-2 Virus **Envelope** Protein Produced in Baculovirus Infected Insect Cells. Arch. Virol. 1994, 139.197-207.
DETD 16. Cole, G. A., Charles, J., Wisseman, L. Pathogenesis of Type 1 **Dengue** Virus Infection in Suckling, Weaning and Adult Mice. Am. J. Epidemiol. 1969, 89,669-680.
DETD . . . R. Putnak, Walter Reed Army Institute of Research, Washington, DC and its sequence is listed in Irie, K., Mohan, P. **M**., Sasaguri, Y., Putnak, R., Padmanabhan, R., Sequence analysis of cloned **dengue** virus type 2 genome (New Guinea-C strain), Gene &5(2): 197-211 (Feb 20, 1989).
DETD . . . W. S., Biedrzycka, A., Wright, P. J. Partial Nucleotide Sequence and Deduced Amino Acid Sequence of the Structural Proteins of **Dengue** Virus Type 2, New Guinea C and PUO-218 Strains. J. Gen. Virol. 1988, 69, 1391-1398.
DETD 20. Sambrook, J., Fritsch, **E**., Manniatis, T. Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.
DETD 22. Laemmli, **E**. K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:680-685.
DETD 23. Raz, **E**., Carson, D., Parker, S., Parr, T., Abai, A., Aichinger, G., Gromkowski, S., Singh, **M**., Lew, D., Yankauckas, **M**., Baird, S., Rhodes, G. Intradermal Gene Immunization: The Possible Role of DNA Uptake in the Induction of Cellular Immunity to. . .
DETD 24. Ansari, **M**., Shope, R., Malik, S. Evaluation of vero cell lysate antigen for the ELISA of **flaviviruses**. J. Clin. Lab. 1993, Anal. 7: 230-237.
DETD 25. Eckels, K., Harrison, V., McCown, J., Russel, P. Isolation of Temperature Sensitive **Dengue** 2 Virus Under Conditions Suitable for

Vaccine Development. Infect. Immun. 1976, 14, 1221-1227.
DETD
NUMBER OF SEQUENCES: 24
SEQUENCE CHARACTERISTICS:
LENGTH: 2357 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULE TYPE: RNA (genomic)
HYPOTHETICAL?: NO
ANTI-SENSE?: NO
FRAGMENT TYPE: internal
ORIGINAL SOURCE:
ORGANISM: **Dengue** virus
STRAIN: New Guinea C
POSITION IN GENOME:
CHROMOSOME/SEGMENT NAME/NUMBER: PreM and **Envelope**
MAP POSITION: 330-2446
POSITION UNITS: bp
PUBLICATION INFORMATION:
AUTHORS: Gruenberg, A Woo, W S Biedrzycka, A
Wright, P J
TITLE: Partial nucleotide sequence and deduced amino acid
sequence of the structural proteins of **dengue** virus
type 2, New Guinea C and PUO-218 strains
JOURNAL: J. Gen. Virol.
VOLUME: 69
PAGES: 1391-1398
DATE: 1988
PUBLICATION INFORMATION:
AUTHORS: Irie, K Mohan, P **M** Sasaguri, Y
Putnak, R Padmanabhan, R
TITLE: Sequence Analysis of Cloned **dengue** virus type 2 genome
(New Guinea-C strain)
JOURNAL: Gene
VOLUME: 75
ISSUE: 2
PAGES: 197-211
DATE: 1989
PUBLICATION INFORMATION:
AUTHORS: Yaegashi, T Vakharia, V N Page, K. . .
2357
SEQUENCE CHARACTERISTICS:
LENGTH: 1941 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
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LOCATION: 34..531
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NAME/KEY: misc_feature
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/evidence= EXPERIMENTAL
FEATURE:

NAME/KEY: misc_signal
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CCACACATGA TCGTCAGTAG ACAAGAGAAA GGGAAAAGTC TTCTGTTTAA AACAGAGGAT 120
GGTGTGAACA TGTGTACCCT CATGGCCATG GACCTTGGTG AATTGTGTGA AGATACAATC. . . 1941
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NAME/KEY: misc_feature
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FEATURE:
NAME/KEY: misc_signal
LOCATION: 1..33
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OTHER INFORMATION: /function= "**Signal sequence**" /evidence= EXPERIMENTAL
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CCACACATGA TCGTCAGTAG ACAAGAGAAA GGGAAAAGTC TTCTGTTTAA AACAAAGGAT 120
GGTGTGAACA TGTGTACCCT CATGGCCATG GACCTTGGTG AATTGTGTGA AGATACAATC. . .
TGTATTTGGG AGTTATGGTG CAGGCC 2016
SEQUENCE CHARACTERISTICS:
LENGTH: 2042 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
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NAME/KEY: misc_feature
LOCATION: 1..59
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OTHER INFORMATION: /product= "PreM **Signal Sequence**"
/evidence= EXPERIMENTAL
FEATURE:
NAME/KEY: misc_feature
LOCATION: 60..497
IDENTIFICATION METHOD: experimental
OTHER INFORMATION: /product= "PreM" /evidence= EXPERIMENTAL
FEATURE:
NAME/KEY: misc_feature
LOCATION: 498..2042
IDENTIFICATION METHOD: experimental
OTHER INFORMATION: /product= "**Envelope**" /evidence= EXPERIMENTAL
PUBLICATION INFORMATION:
AUTHORS: Mason, P W McAda, P C Mason, T L
Fournier, **M** J
TITLE: Sequence of the **dengue**-1 virus genome in the region
encoding the three structural proteins and the major
nonstructural protein NS1
JOURNAL: Virology
VOLUME: 161

PAGES: 252-267
RELEVANT RESIDUES IN SEQ ID NO: 4 FROM 1 TO 2400
PUBLICATION INFORMATION:
AUTHORS: Fu, J Tan, B H Yap, **E** H
Chan, Y C Tan, Y H
TITLE: Full-length cDNA sequence of **dengue** type-1 virus
(Singapore strain S275/90)
JOURNAL: Virology
VOLUME: 188
ISSUE: 2
PAGES: 953-958
DATE: 1992
RELEVANT RESIDUES IN SEQ ID NO: 4 FROM 1 TO 10984
SEQUENCE:. . . 1980
TTTCAATGAC GTGTATCGCA GTTGGCATGG TCACGCTGTA CCTAGGAGTC ATGGTTCAGG 2040
CG 2042
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TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear
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FEATURE:
NAME/KEY: misc_feature
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FEATURE:
NAME/KEY: misc_feature
LOCATION: 498..2049
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/standard_name= "**Envelope** protein"
PUBLICATION INFORMATION:
AUTHORS: Osatomi, K Fuke, I Tsuru, D
Shiba, T Sakaki, Y Sumiyoshi, H
TITLE: Nucleotide sequence of **dengue** type 3 virus genomic RNA
encoding viral structural proteins
JOURNAL: Virus Genes
VOLUME: 2
ISSUE: 1
PAGES: 99-108
DATE: 1988
PUBLICATION INFORMATION:
AUTHORS: Osatomi, K Sumiyoshi, H
TITLE: Complete nucleotide sequence of **dengue** 3 virus genome
RNA
JOURNAL: Virology
VOLUME: 176
ISSUE: 2
PAGES: 643-647
DATE: 1990
SEQUENCE: 5
ATGCTGAGCA TTATCAACAA ACGGAAAAAG ACATCGCTCT GTCTCATGAT GATGTTACCA 60
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GGGGGTCGTG 2040
GTGCAAGCT 2049
SEQUENCE CHARACTERISTICS:
LENGTH: 2052 base pairs
TYPE: nucleic acid
STRANDEDNESS: single
TOPOLOGY: linear

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/evidence= EXPERIMENTAL
FEATURE:
NAME/KEY: misc_feature
LOCATION: 73..569
IDENTIFICATION METHOD: experimental
OTHER INFORMATION: /product= "PreM" /evidence= EXPERIMENTAL
FEATURE:
NAME/KEY: misc_feature
LOCATION: 570..2052
OTHER INFORMATION: /product= "**Envelope**"
PUBLICATION INFORMATION:
AUTHORS: Zhao, B Mackow, **E** Buckler,
White, A
TITLE: Cloning full length **dengue** type-4 viral DNA sequences
analysis of genes coding for structural proteins
JOURNAL: Virology
VOLUME: 155
ISSUE: 1
PAGES: 77-88
DATE: 1986
SEQUENCE: 6
ATGCTGAACA TCTTGAATGG GAGAAAAAGG TCAACAATGA. . .
. . . form, wherein said plasmid expression vector is functional in mammalian subjects and includes preM and at least 92% of the **envelope** gene of a **dengue** W virus, where W is a number selected from the group consisting of 1, 2, 3 and 4.

. . . The pharmaceutical composition of claim 1 further comprising a second plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** X virus, where X is a number different from W and is selected from a the group consisting of 1,. . .
. . . The pharmaceutical composition of claim 2 further comprising a third plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** Y virus, where Y is a number different from W and from X and is selected from the group consisting. . .
. . . The pharmaceutical composition of claim 3 further comprising a fourth plasmid including the PreM and at least 92% of the **envelope** gene of **dengue** Z virus, where Z is a number different from W, from X, and from Y, and is selected from the. . .

L14 ANSWER 3 OF 15 USPATFULL on STN
2002:167888 Recombinant nonstructural protein subunit vaccine against **flaviviral** infection.
McDonell, Michael, Kailua, HI, United States
Peters, Iain, Honolulu, HI, United States
Coller, Beth-Ann, Aiea, HI, United States
Hawaii Biotechnology Group, Inc., Aeia, HI, United States (U.S. corporation)
US 6416763 B1 20020709
APPLICATION: US 1998-143077 19980828 (9) <--
DOCUMENT TYPE: Utility; GRANTED.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The recombinant expression and secretion from eucaryotic host cells, particularly Drosophila cells, of **Flavivirus** nonstructural (NS) protein, particularly NS1, is useful in combination with **Flavivirus** truncated **envelope** (**E**) protein to protect a host subject from infection and disease from **Flavivirus** species. Further, NS1 is useful as a diagnostic of **flaviviral** infection.

Compositions of truncated **flaviviral envelope** protein and **flaviviral** nonstructural protein induce high titer virus neutralizing

antibodies believed to be important in protection against **flavivirus** infection and which are useful in diagnosis of infection by the virus.

LM What is claimed is:

1. An immunogenic composition which induces an immunological response in a host subject inoculated with said composition comprising a carrier and a mixture comprising a **Flavivirus** truncated **envelope** (**E**) protein and a **Flavivirus** nonstructural (NS) protein, wherein said nonstructural protein (NS) protein has been secreted as a recombinantly produced protein, from Drosophila cells, and wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

2. The immunogenic composition of claim 1, wherein said **envelope** protein (**E**) protein has been secreted as a recombinantly produced protein from Drosophila cells.

3. The immunogenic composition of claim 1 wherein the non-structural (NS) protein is encoded in a DNA construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA_L).

4. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.

5. The immunogenic composition of claim 1 wherein said Drosophila cells are D. melanogaster Schneider cells.

6. The immunogenic composition of claim 2 wherein said Drosophila cells are D. melanogaster Schneider cells.

7. A method to produce an immunogenic composition comprising (a) culturing the Drosophila cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said nonstructural (NS) protein of the **Flavivirus** strain against which enhanced protection is sought; (b) recovering the nonstructural (NS) protein from the culture medium; and (c) combining said NS with a **Flavivirus** truncated **envelope** protein, wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

8. A method to produce an immunogenic composition comprising (a) culturing the Drosophila cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS1) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said NS1 protein of the **Flavivirus** strain against which enhanced protection is sought; (b) recovering the NS1 protein from the culture medium; and (c) combining said NS1 with a **Flavivirus** truncated **envelope** protein, wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

9. The immunogenic composition of claim 1, wherein the nonstructural (NS) protein is NS1.

10. An immunodiagnostic for the detection of a **Flavivirus**, wherein said immunodiagnostic comprises, the immunogenic composition of claim 1.

11. The immunodiagnostic of claim 10, wherein the nonstructural (NS) protein is NS1.

12. The immunodiagnostic of claim 10, wherein said **Flavivirus** is a **dengue** virus.

13. The immunodiagnostic of claim 10, wherein said **envelope** (**E**) protein has been secreted as a recombinantly produced protein from Drosophila cells.

14. The immunodiagnostic of claim 13, wherein said Drosophila cells are D. melanogaster Schneider cells.

TI Recombinant nonstructural protein subunit vaccine against **flaviviral** infection

AI US 1998-143077 19980828 (9) <--

AB The recombinant expression and secretion from eucaryotic host cells, particularly Drosophila cells, of **Flavivirus** nonstructural (NS) protein, particularly NS1, is useful in combination with **Flavivirus** truncated **envelope** (**E**) protein to protect a host subject from infection and disease from **Flavivirus** species. Further, NS1 is useful as a diagnostic of **flaviviral** infection.

AB Compositions of truncated **flaviviral envelope** protein and **flaviviral** nonstructural protein induce high titer virus neutralizing antibodies believed to be important in protection against **flaviviral** infection and which are useful in diagnosis of infection by the virus.

SUMM This invention relates to protection against and diagnosis of **flaviviral** infection. More specifically, this invention concerns recombinantly produced subunits of a nonstructural **flaviviral** protein that is expressed and secreted as a mature polypeptide from eucaryotic cells. Compositions of truncated **flaviviral envelope** protein in combination with **flaviviral** nonstructural protein induce a higher degree of protection against **flaviviral** infection than the truncated protein alone. These compositions may be useful in the prevention, diagnosis or treatment of **flaviviral** infection. The present invention relates to compositions of matter and methods of making and methods of using said compositions as. . .

SUMM The family **Flaviviridae** includes the Japanese **encephalitis virus** (JE), Tick-borne **encephalitis virus** (TBE), West Nile virus (WN), **dengue** virus (including the four serotypes of: DEN-1, DEN-2, DEN-3, and DEN4), and the family prototype, **yellow fever virus** (YF). In the case of **dengue**, the viruses are transmitted to man by mosquitoes of the genus Aedes, primarily A. aegypti and A. albopictus. The viruses. . . headache, aching muscles and joints, and rash. Some cases, typically in children, result in a more severe forms of infection, **dengue** hemorrhagic fever and **dengue** shock syndrome (DHF/DSS), marked by severe hemorrhage, vascular permeability, or both, leading to shock. Without diagnosis and prompt medical intervention,. . .

SUMM **Flaviviruses** are the most significant group of arthropod-transmitted viruses in terms of global morbidity and mortality with an estimated one hundred million cases of **dengue** fever occurring annually Halstead, S. B. 1988. Pathogenesis of **Dengue**: Challenges to Molecular Biology Science 239:476-481. With the global increase in population and urbanization especially throughout the tropics, and the lack of sustained mosquito control measures, the mosquito vectors of **flavivirus** have distributed throughout the tropics, subtropics, and some temperate areas, bringing the risk of **flaviviral** infection to over half the world's population. Modern jet travel and human emigration have facilitated global distribution of **dengue** serotypes, such that now multiple serotypes of **dengue** are endemic in many regions. Accompanying this in the last 15 years has been an increase in the frequency of **dengue** epidemics and the incidence of DHF/DSS. For example, in Southeast Asia, DHF/DSS is a leading cause of hospitalization and death. . .

SUMM **Flaviviruses** are small, enveloped viruses containing a single, positive-strand, genomic RNA, approximately 10,500 nucleotides in length

containing short 5' and 3' . . . a single long open reading frame, a 5' cap, and a nonpolyadenylated 3' terminus. The complete nucleotide sequence of numerous **flaviviral** genomes, including all four DEN serotypes and YF virus have been reported (Fu, J. et al., 1992 Virology 188:953-958; Deubel,. . . 1986, Virology 155:365-377; Hahn, Y. S. et al., 1988, Virology 162:167-180; Osatomi, K. et al., 1990, Virology 176:643-647; Zhao, B. **E**. et al., 1986, Virology 155:77-88; Mackow, **E**. et al., 1987, Virology 159:217-228; Rice, C. **M**. et al., 1985, Science 229:726-733). All **flaviviral** proteins are derived from a single long polyprotein through precise processing events mediated by host as well as virally encoded. . . gene products encoded by the single open reading frame are translated as a polyprotein organized in the order, capsid (C), `**preMembrane**` (**prM**, which is processed to `**Membrane**` (**M**) just prior to virion release from the cell) and `**envelope** (**E**)`; following this are the non-structural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (reviewed in Chambers, Thomas J., Chang S. Hahn, Ricardo Galler, and Charles **M**. Rice. 1990. **Flavivirus** Genome Organization, Expression, and Replication. Ann. Rev. Microbiol. 44: 649-688; Henchal, Erik A., and J. Robert Putnak. 1990. The **Dengue** Virus. Clin. Microbiol. Rev. 3 (4): 376-396). A stretch of hydrophobic residues at the C-terminal end of **E** serve both as its **membrane** anchor as well as **signal sequence** directing NS1 for translocation into the endoplasmic reticular lumen. Thus precise cleavage at the **E**-NS1 junction is provided by host signal peptidase Falgout, B., R. Chanock, and C.-J. Lai. 1989. Proper Processing of **Dengue** Virus Nonstructural Glycoprotein NS1 Requires the N-Terminal Hydrophobic **Signal Sequence** and the Downstream Nonstructural Protein NS2a. J. Virol. 63: 1852-60), while the virally-encoded protease NS2a is responsible for processing at the NS1 C-terminus Leblois, H., and P. R. Young. 1995. Maturation of the **Dengue**-2 Virus NS1 Protein in Insect Cells: Effects of Downstream NS2A Sequences on Baculovirus-expressed Gene Constructs. J. Gen. Virol. 76: 979-984).. . . replication of viral RNA is suggested by immunolocalization studies which demonstrate its association with the replicative form dsRNA Mackenzie, J. **M**., **M**. K. Jones, and P. R. Young. 1996. Immunolocalization of the **Dengue** Virus Nonstructural Glycoprotein NS1 Suggests a Role in Viral RNA Replication. Virol. 220: 232-240) as well as blockage of RNA accumulation by a temperature-sensitive NS1 mutation Muylaert, I. R., R. Galler, and C. **M**. Rice. 1997. Genetic Analysis of the **Yellow Fever Virus** NS1 Protein: Identification of a Temperature-Sensitive Mutation which Blocks RNA Accumulation. J. Virol 71: 291-98). Further studies utilizing gene complementation. . . role in RNA replication to be just prior to or at initiation of minus-strand synthesis Lindenbach, B. D. and C. **M**. Rice. 1997. Trans-Complementation of **Yellow Fever Virus** NS1 Reveals a Role in Early RNA Replication. J. Virol 71: 9608-17). Meanwhile, work of others has indicated that the. . . polymerase activity necessary for viral nucleic acid replication is provided by NS5 Tan, B. H., J. Fu, R. J. Sugrue, **E**. H. Yap, Y. C. Chan and Y. H. Tan. 1996. Recombinant **Dengue** Type 1 Virus NS5 Protein Expressed in Escherichia coli Exhibits RNA-Dependent RNA Polymerase Activity. Virology 216: 317-25).

SUMM . . . comparing the nucleotide sequence and the amino terminal sequences of the viral proteins. Subsequent to initial processing of the polyprotein, **prM** is converted to **M** during viral release (Wengler, G. et al., 1989. J. Virol 63:2521-2526) and anchored C is processed during virus maturation (Nowak et al., 1987. Virology 156:127-137). The **envelope** of **flaviviruses** is derived from the host cell **membrane** and is decorated with virally-encoded transmembrane proteins **membrane** (**M**) and **envelope** (**E**). While mature **E** protein and the precursor to **M**, **prM**, are glycosylated, the much smaller mature **M** protein is not. The **E** glycoprotein, which is the largest viral structural protein, contains functional domains responsible for cell surface attachment and intraendosomal fusion activities.. . .

SUMM While all **dengue** viruses are antigenically related, antigenic distinctions exist that define the four **dengue** virus serotypes. Infection of an individual with one serotype does not apparently provide

long term immunity against the other serotypes. In . . against type-specific determinants. On the other hand, secondary infection by a heterologous serotype is characterized by IgG antibodies that are **flavivirus** crossreactive. Consecutive infection with different serotypes is thought to be a major factor contributing to DHF.

SUMM Many studies have established the effectiveness of immunoprophylaxis with properly folded **flavivirus envelope** protein in the prevention of disease in several host subject animal models. However, vaccination against **dengue** is complicated by observations of enhanced infection associated with the presence of virion-reactive antibodies at sub-neutralizing concentrations or of non-neutralizing. . . (ADE) pathway is thought to account for high incidence of the often fatal hemorrhagic fever and shock syndrome forms of **dengue** occurring in children possessing immunity to a **dengue** serotype not matching the current infection (Halstead, '88 supra). This has prompted several workers to investigate the potential of **Flaviviridae** vaccines based on nonstructural proteins, since antibodies reactive against these viral proteins are unlikely to enhance virion entry into monocytes. . . domain receptors, the suspected route of ADE (Halstead, '8 supra). Immunization with NS1 has yielded variable degrees of protection against **flavivirus** infection in mouse and monkey disease models (see Table 1). However, there are few studies comparing immunization with NS1 in combination with **envelope** (**E**) protein, particularly with truncated **envelope** (**E**) protein.

SUMM . . . on the surface of virally-infected cells (Smith, G. W., and P. J. Wright. 1985. Synthesis of Proteins and Glycoproteins in **Dengue** Type 2 Virus-Infected Vero and Aedes Albopictus Cells. J. Gen. Virol. 66: 559-71) and immunoprophylaxis appears to be due to T-lymphocyte killing (**e**.g. Hall, R. A., T. N. H. Brand, **M**. Lobigs, **M**. Y. Sangster, **M**. J. Howard, and J. S. Mackenzie. 1996. Protective Immune Responses to the **E** and NS1 Proteins of Murray Valley **Encephalitis Virus** in Hybrids of **Flavivirus**-Resistant Mice. J. Gen. Virol. 77: 1287-94 Jacobs, S. C., J. R. Stephenson and G. W. G. Wilkinson. 1994. Protection Elicited. . . by Replication-Defective Adenovirus Vector and Aedes Albopictus Cells. J. Gen, Virol 66: 559-71) and/or complement-mediated cytolysis (Schlesinger, J. J., **M**. W. Brandriss, J. R. Putnak, and **E**. **E**. Walsh. 1990. Cell Surface Expression of **Yellow Fever Virus** Non-structural Glycoprotein NS1: Consequences of Interaction with Antibody. J. Gen. Virol. 71: 593-99 Schlesinger, J. J., **M**. Foltzer, and S. Chapman. 1993. The Fc Portion of Antibody to **Yellow Fever Virus** NS1 is a Determinant of Protection against YF Encephalitis in Mice. Virol. 192: 132-141 Lin, Y.-L., L.-K. Chen, C.-L. Liao, C.-T. Yeh, S.-H. Ma, J.-L. Chen, Y.-L. Huang, S.-S. Chen and H.-Y. Chiang. 1998. DNA Immunization with Japanese **Encephalitis Virus** Nonstructural Protein NS1 Elicits Protective Immunity in Mice. J. Virol. 72: 191-200) facilitated by NS1-reactive antibodies. In some cases at least, it appears that poor protection is associated with rapidly replicating **flaviviruses** and may be due to a relatively short window of opportunity for destruction of infected cells prior to virion release. For example, Falgout, B., R. Chanock, and C. -J. Lai. 1989. Proper Processing of **Dengue** Virus Nonstructural Glycoprotein NS1 Requires the N-Terminal Hydrophobic **Signal Sequence** and the Downstream Nonstructural Protein NS2a. J. Virol. 63: 1852-60) were unable to get good protection against DEN-4 despite using. . . complete protection against DEN-2; they attribute this to the slower replication rate of the latter. Also Cane, P. A., and **E**. A. Gould. 1988. Reduction of **Yellow Fever Virus** Mouse Neurovirulence by Immunization with a Bacterially Synthesized Non-structural Protein (NS1) Fragment. J. Gen. Viro. 69: 1241-46 were able to. . . obtain significant protection against a slow growing strain, but not more virulent strains of Yellow Fever (YF), following immunization with **E**. coli-expressed YF NS1. However, mouse strain and gender also seem to be important, as Qu, X., W. Chen, T. Maguire, and F. Austin. 1993. Immunoreactivity and Protective Effects in Mice of a Recombinant **Dengue** 2 Tonga Virus NS1 Protein Produced in a Baculovirus Expression System. J. Gen. Virol. 74: 89-97were able to get reasonable. . .

SUMM . . . JEV NS1 provided a higher level of protection (90% vs. 70%) than obtained with an analogous construct directing expression of **prM** and **E** proteins (Lin et al., '9 supra). Clearly different mechanisms mediate the immunoprophylactic activities of viral structural and nonstructural proteins and. . .

SUMM . . . 17D virus. al, '85**

17D virus Protection: all 11 NS1-immunized mice survived vs. 2 of 10 control mice mock-immunized with ovalbumin.

E. coli expressed Yellow Fever 17D NS1- Mouse challenge: intracerebral injection of Cane and

β-galactosidase fusion; produced as Yellow Fever 17D RMP. . . Protection: 17 of 20 mice were protected vs. 1/18 for control group.

Baculovirus expressed Japanese Mouse challenge: intraperitoneal injection McCown et al,

Encephalitis Virus (JEV) NS1, crude cell of JEV. '90 infra

lysate (note that proper processing at the Protection[a]: not significantly better than

NS1-NS2A junction. . . conformation of NS1.

[b]these results were quite dependent on mouse strain and gender, see text for details.

*Schlesinger, J. J., **M**. W. Brandriss, C. B. Cropp, and T. P. Monath, 1986. Protection against Yellow Fever in Monkeys by Immunization with **Yellow Fever Virus** Nonstructural Protein NS1. J. Virol. 60; 1153-55

Schlesinger, J. J., **M. W. Brandriss, and **E**. **E**. Walsh, 1985. Protection against 17D Yellow Fever Encephalitis in Mice by Passive Transfer of Monoclonal Antibodies to the Nonstructural Glycoprotein. . .

SUMM Schlesinger, J. J., **M**. W. Brandriss, C. B. Cropp, and T. P. Monath. 1986. Protection against Yellow Fever in Monkeys by Immunization with **Yellow Fever Virus** Nonstructural Protein NS1. J. Virol. 60: 1153-55--and on next line, please insert--** Schlesinger, J. J., M. **W**. Brandriss, and E. **E**. **Walsh**. 1985. Protection against 17D Yellow Fever Encephalitis in Mice by Passive Transfer of Monoclonal Antibodies to the Nonstructural Glycoprotein gp48. . .

SUMM Despite uncertainty regarding the exact mechanism of NS1-mediated immunoprophylaxis against **flavivirus** infection, it is clearly different from the virus neutralizing activity provided by α-**envelope** antibodies since little of the NS1 protein is present on the viral surface. It is reasonable, therefore, to suspect that NS1 may augment effectiveness of **flavivirus** vaccines based on recombinant viral **E** protein by providing a second route of protection to that afforded by an immunological response against **E** protein. However, there have been relatively few studies that directly compare the protective properties of immunogens composed of **flavivirus envelope** protein in isolation versus in combination with NS1. McCown, Jack, Mark Cochran, Robert Putnak, Robert Feighny, Jeanne Burrous, Erik Henchal,. . . a Recombinant Baculovirus Vaccine. Am. J. Trop. Med Hygiene. 42 (No. 5): 491-499) tested crude cell lysate Schlesinger, J. J., **M**. W. Brandriss, and **E**. **E**. Walsh. 1987. Protection of Mice against **Dengue** 2 Virus Encephalitis by Immunization with the **Dengue** 2 Virus Non-structural Glycoprotein NS1. J. Gen. Virol. 68: 853-57 immunogens prepared from baculovirus constructs based on JEV **E** or NS1 individually as well as a polyprotein containing **prM/M**, **E**, NS1 and NS2a/b. Fifteen of twenty mice immunized with **E** were protected from a subsequent intraperitoneal challenge of JEV, while protection was 13/19 in the case of **prM**-NS2 polyprotein; the lack of improvement in protection by including NS1 is perhaps not surprising since immunization with this protein alone. . . yielded some protection against DEN-4 viremia following immunization with a crude lysate from insect cells infected with a baculovirus DEN-4 C-**prM**-**E**-NS1-NS2a polyprotein construct, but parallel immunization with baculovirus-expressed **E** gave a similar level of protection (**E** alone: no viremia in 1 of 3 animals; C-NS2a polyprotein: 1 of 6 showed no viral growth, while another had viremia of reduced duration. Lai, C.-J., Y.-**M**. Zhang, R. Men, **M**. Bray, R. **M**. Chanock, D. R. Dubois, and K. H. Eckels. 1990.

Immunization of Monkeys with Baculovirus Recombinant Expressed **Dengue** **Envelope** and NS1 Glycoproteins Induces Partial Resistance to Challenge with Homotypic **Dengue** Virus. In Vaccines 90, edited by F. Brown, R. **M.** Chanock, H. S. Ginsberg and R. A. Lerner. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. Two other studies of mouse protection provided by **E** vs. **E**+NS1 immunizations, were done under conditions that yielded complete protection with **E** alone and thus allowed little opportunity for augmentation by NS1 (JEV vaccinia **prM-E** vs. **prM**-NS2a: Konishi, **E.**, S. Pincus, B. Fonseca, R. Shope, **E.** Paoletti, and P. Mason. 1990. Comparison of Protective Immunity Elicited by Recombinant Vaccinia Viruses that Synthesize **E** or NS1 of Japanese **Encephalitis Virus**. Virology 185: 401-410; DBN-4 vaccinia C-B vs. C-NS2a: Bray, **M**,, B. Zhao, L. Markoff, K. H. Eckels, R. **M.** Chanock, and C.-J. Lai. 1989. Mice Immunized with Recombinant Vaccinia Virus Expressing **Dengue** 4 Virus Structural Proteins with or without Nonstructural Protein NS1 are Protected against Fatal **Dengue** Virus Encephalitis. J. Virol. 63: 2853-56), although it appears that the level of protection observed may be largely dependent upon parameters of the animal model used. The art contains few clear examples of controlled comparisons between use of truncated **envelope** protein alone and in combination with nonstructural protein, NS1, to stimulate a protective response. The studies of McCown et al. ('90) and Feighny, Robert, Jeanne Burrous, Jack McCown, Charles Hoke, and Robert Putnak. 1992. Purification of Native **Dengue**-2 Viral Proteins and the Ability of Purified Proteins to Protect Mice. Am. J. Trop. Med Hygiene 47 (No. 4): 405-412). . .

SUMM The invention provides immunogenic compositions containing, as an active ingredient, a secreted recombinantly produced nonstructural (NS) protein of a **Flavivirus**. The invention further provides immunogenic compositions containing as a second active ingredient, a secreted recombinantly produced **Flavivirus** truncated **envelope** protein (**E**). These immunogenic compositions are capable of eliciting the production of neutralizing antibodies against a **Flavivirus**. In the illustrations below, the nonstructural protein NS1 from **dengue** virus, a **Flavivirus**, is recombinantly expressed and secreted from Drosophila host cells. Similarly expressed is the truncated **envelope** protein (**E**). Together, NS1 and **E** serve to protect mice challenged with infection by **dengue** virus.

SUMM . . . is drawn to methods of the recombinant expression and secretion from eucaryotic host cells of nonstructural (NS) protein subunits of **Flavivirus**. One embodiment of this invention relates to the methods of recombinant expression and secretion from Drosophila host cells of the NS1 protein of **Flavivirus**. Further, this invention contemplates methods of the recombinant expression and secretion of other nonstructural proteins of **Flavivirus** using other vectors, control sequences, secretory signal sequences as well as other eucaryotic host cells.

SUMM Another aspect of the present invention relates to the use of compositions of a **Flavivirus** truncated **envelope** (**E**) protein in combination with nonstructural proteins of **Flavivirus**, as immunogenic antigens that stimulate an immunological response in a host subject animal, inter alia, by stimulating antibody formation and/or a cellular immune response. One embodiment of this invention includes an immunogenic composition of matter comprising the **Flavivirus** truncated **envelope** (**E**) protein, 80%**E**, and the **Flavivirus** nonstructural (NS) protein, NS1.

SUMM . . . acceptable carrier as a pharmaceutical composition; and use of the immunogenic composition as an immunodiagnostic for the detection of a **Flavivirus**. The invention envisions such immunodiagnostics as using the immunogenic composition as an antigen as well as immunodiagnostics employing antibodies elicited. . .

SUMM Still other aspects of this invention include the compositions of nonstructural proteins of **Flavivirus**. These compositions, including NS1, are useful as immunodiagnostics for the detection of **Flavivirus**. Such immunodiagnostics include nonstructural proteins or fragments thereof as immunogenic compositions as well as immunodiagnostics

employing antibodies elicited in response. . .

SUMM . . . The invention provides, for the first time, a means for increasing the protection of a subject against infection by a **Flavivirus**, by including in a vaccine an immunogenic composition that contains a recombinantly expressed **Flavivirus** nonstructural (NS) protein subunit secreted from a eucaryotic host cell. The DNA sequence encoding a nonstructural (NS) protein is obtained from a **Flavivirus**, and expressed following the functional and operable insertion of the DNA sequence into an expression vector containing control sequences and. . .

SUMM . . . DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See **e**.g., Sambrook, Fritsch, and Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989), Oligonucleotide Synthesis (**M**. J. Gait Ed., 1984), Animal Cell Culture (R. I. Freshhey, Ed., 1987), the series Methods in Enzymology (Academic Press, Inc.); Gene Transfer Vectors for Mammalian Cells (J. **M**. Miller and **M**. P. Calos eds. 1987), Handbook of Experimental Immunology, (D. **M**. Weir and C. C. Blackwell, Eds.), Current Protocols in Molecular Biology (F. **M**. Ausubel, R. Brent, R. **E**. Kingston, D. D. Moore, J. G. Siedman, J. A. Smith, and K. Struhl, eds., 1987), and Current Protocols in Immunology (J. **E**. Coligan, A. **M**. Kruisbeek, D. H. Margulies, **E**. **M**. Shevach and W. Strober, eds., 1991). All patents, patent applications, and publications mentioned herein, both supra and infra, are hereby. . .

SUMM For example, **Flavivirus** nonstructural (NS) proteins may include: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Chambers et al, '90 supra; Henchal and. . .

SUMM Such methods contemplated by this invention include a method of expressing a **Flavivirus** nonstructural (NS) protein, comprising, transforming a eucaryotic host cell with a recombinant vector comprising a DNA sequence encoding a **Flavivirus** nonstructural (NS) protein; cultivating the transformed host under permissive conditions for expression and secretion of the nonstructural protein (NS); and. . .

SUMM . . . embodiment of this invention relates to the recombinant expression and secretion from Drosophila host cells of the NS1 protein of **Flavivirus**. Further, this invention contemplates the recombinant expression and secretion of other nonstructural proteins of **Flavivirus** using other vectors, control elements, signal or secretion elements as well as other eucaryotic host cells.

SUMM Another embodiment of the present invention relates to the use of compositions of a **Flavivirus** truncated **envelope** (**E**) protein in combination with nonstructural proteins of **Flavivirus**; as immunogenic antigens that stimulate an immunological response in a host subject, inter alia, by eliciting antibody formation and/or a cellular immune response. A more specific embodiment of this invention includes an immunogenic composition of matter comprising the **Flavivirus** truncated **envelope** (**E**) protein, 80%**E**, and the **Flavivirus** nonstructural (NS) protein, NS1. The recombinant product we have focused most of our efforts on is a soluble form of **flaviviral E**, which is truncated at the carboxy-terminal end resulting in a polypeptide which represents approximately 80% of the full-length **E** molecule (amino acids 1-395; 80%**E**).

SUMM The recombinant expression and secretion of **Flavivirus** truncated **envelope** (**E**) protein was carried out. The construction of recombinantly expressed **Flavivirus** truncated **envelope** (**E**) protein secreted from eucaryotic host cells has been thoroughly presented in copending patent application by Peters et al., Ser. No.. . .

SUMM . . . from PCR amplification of a sequence from DEN-2 PR159/S1 cDNA clone #2 (Hahn, Young S., Ricardo Galler, Tim Hunkapiller, Joel **M**. Dalrymple, James H. Strauss, and Ellen G. Strauss. 1988. Nucleotide Sequence of **Dengue** 2 RNA and Comparison of the Encoded Proteins with Those of Other **Flaviviruses**. Virology 162: 167-180; pYH2, provided by J. R. Putnak, Walter Reed Army Institute of Research) using primers designed to add. . . the sequences encoding the proteins to be expressed are operably linked to control sequences and secretory signal sequences. The truncated **E** protein may be expressed separately or

fused to NS1.

SUMM "Secretory **signal sequence**" refers to a peptide sequence, encoded by a DNA sequence or sequences, which are capable when the DNA sequence or. . . signal peptide encoded by the DNA sequence is thought to be important for targeting the synthesized polypeptide for secretion. A **signal sequence** plays an important role in ensuring the proper localization of a newly synthesized protein. Generally they provide "topogenic signals" (Blobel,. . .

SUMM . . . such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMT-II (Karin, **M**., et al., Nature (1982) 299:797-802) may also be used. General aspects of mammalian cell host system transformations have been described. . . compatible and operable for use in each of these host types as well as are termination sequences and enhancers, as **e**.g. the baculovirus polyhedrin promoter. As above, promoters can be either constitutive or inducible. For example, in mammalian systems, the MTII. . .

SUMM . . . walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology (1978) 52:546, optionally as modified by Wigler, **M**., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, **E**. Lacey, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes. Cell. . .

SUMM . . . in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, **e**.g., New England Biolabs, Product Catalog. In general, about 1 µg of plasmid or DNA sequence is cleaved by one unit. . . in Methods in Enzymology (1980) 65:499-560. Restriction cleaved fragments may be blunt ended by treating with the large fragment of **E**. coli DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15. . . to 25° C. in 50 mM Tris pH 7.6, 50 mM NaCl, 6 mM $MgCI_2$, 6 mM DTT and 5-10 µ**M** dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the. . .

SUMM Immunogenic compositions containing **Flavivirus** nonstructural (NS) proteins or truncated **envelope** (**E**) proteins to be used as antigens are prepared and utilized in ways that the skilled artisan would readily recognize. Antigens. . .

SUMM . . . Freunds Adjuvant (CFA) and Incomplete Freunds Adjuvant (IFA); (5) cytokines, such as interleukins (IL-1, IL-2, etc.), macrophage colony stimulating factor (**M**-CSF), tumor necrosis factor (TNF), etc; and (6) other substances that act as immunostimulating agents to enhance the effectiveness of the. . .

SUMM The immunogenic compositions (**e**.g., the antigen, pharmaceutically acceptable carrier, and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary. . . carriers. Immunogenic compositions of the present invention elicit formation of antibodies with high binding specificity to a composition of a **Flavivirus** truncated **envelope** (**E**) protein in combination with nonstructural proteins of **Flavivirus**, and more specifically antibodies with high binding specificity to a composition of a **Flavivirus** 80%**E** and NS1. Such immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as. . . physical condition of the individual or host subject animal to be treated, the taxonomic group of individual to be treated (**e**.g., nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the. . . fall in a relatively broad range that can be determined through routine trials. The immunogenic compositions are conventionally administered parenterally, **e**.g., by injection, either subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories,. . .

DRWD FIG. 1 shows the PCR primers used to clone NS1. Upper case represents regions homologous to **dengue** with the reading frame indicated, adapter sequences are in lower case. The first capitalized codon of 0.2422 p codes for Asp_1 of NS1; the endpoint specified by 0.3477 **m** differs

from that identified by Hahn et al (66 supra, (nucleotide 5666), but is in agreement with the 3' termini identified for multiple other **flaviviruses** (reviewed in Chambers et al, '90, supra).

DRWD . . . blot showing the glycosylation of recombinant and viral NS1's. Western blot of S2 DEN-2 NS1 cell culture medium (Recombinant) and **Dengue** 2-infected $C_{6/36}$ cell pellets (Viral) without glycosidase digestion (-) as well as after endoglycosidase H (**E**) or peptide:N-glycosidase F (P).

DETD . . . is a small-plaque, temperature-sensitive vaccine-candidate strain (Eckels, K. H., V. R. Haarrison, P. L. Summers, and P. K. Russell. 1980. **Dengue**-2 Vaccine: Preparation from a Small-Plaque Virus Clone. Infect. Immun. 27: 175-180) that is identical to the wild type parent, PR159. . .

DETD . . . were cotransfected with pMtt-D2NS1 plus pCoHygro selection,asmid at a weight ratio of 20:1 via the calcium phosphate coprecipitation method (Wigler, **M**., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, **E**. Lacey, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of Mammalian Cells with Genes from Procaryotes and Eucaryotes. Cell 16: 777-785; Gibco BRL, Grand Island, N.Y.). The pCoHygro selection plasmid (van der Straten, A., H. Johansen, **M**. Rosenberg, and R. W. Sweet. 1989. Introduction and Constitutive Expression of Gene Products in Cultured Drosophila Cells using Hygromycin B Selection. Meth. Mol. Cell. Biol. 1: 1-18 SmithKline Beecham) contains the **E**. coli hygromycin B phosphotransferase gene under transcriptional control of the D. melanogaster copia transposable element long terminal repeat and confers. . . supplemented with lipids, yeastolate, and Pluronic F68 (Gibco BRL). Cell-free medium was harvested after 7 days of induction with 200 μ**M** $CuSO_4$.

DETD . . . 4). Consistent with NS1 produced by mammalian cells infected with DEN-2 (Winkler, G., V. B. Randolph, G. R. Cleaves, T. **E**. Ryan, and V. Stoller. 1988. Evidence that the Mature Form of the **Flavivirus** Nonstructural Protein NS1 is a Dimer. Virol. 162: 187-96), endoglycosidase H treatment of recombinant NS1 removed roughly half of the sugar, however DEN-2 NS1 produced in virally-infected $C_{6/36}$ insect cells was fully endoglycosidase H-sensitive. This difference between **flavivirus** NS1 proteins from virally-infected mammalian and mosquito cells has been observed previously (Mason, '89) and is presumably a reflection of. . .

DETD . . . is an oligomeric protein; under completely native conditions its apparent molecular weight is roughly 300 kD (Crooks, A. J., J. **M**. Lee, L. **M**. Easterbrook, A. V. Timofeev, and J. R. Stephenson. 1994. The NS1 Protein of Tick-Borne **Encephalitis Virus** Forms Multimeric Species Upon Secretion from the Host Cell. J. Gen. Virol. 75: 3453-60), but in the presence of detergent. . . also was oligomeric with an apparent molecular weight of .about.300 kD (FIG. 5). SDS-PAGE under non-reducing conditions without sample boiling (i.**e**. conditions of Falconar, A. K. I., and P. R. Young. 1990. Immunoaffinity Purification of Native Dimer Forms of the **Flavivirus** Non-Structural Glycoprotein, NS1 J. Virol. Meth. 30: 325-332) demonstrated that the recombinant NS1 oligomer, like naturally occurring NS1, decomposes to. . .

DETD . . . require autocrine growth factors and grow poorly at low cell densities unless exogenous growth factors are provided. S2 DEN-2 NS1 (i.**e**. cells transformed with pMtt-D2NS1) subcloning was done using either preconditioned cell-free medium or a feeder layer of cells separated from the subclone seed by a **membrane** barrier that is porous to the growth factors, but prevents cell passage (Anopore inserts, NUNC). For the first round of. . . 100 μl/well in a 96-well culture dish. After outgrowth, the small-scale cultures were induced for 7 days with $CuSO_4$ (200 μ**M**, final) and the media spotted onto a nitrocellulose **membrane**. Relative levels of NS1 expression were compared by immunoprobing the dot-blot with α-NS 1 MAb 7E11 and the best expressers. . .

DETD . . . through addition of CO_2 and NaOH; dissolved oxygen was maintained at 50% air saturation. Cell densities at inoculation, CuSO4 (200 μ**M**, final) induction and harvest were 1×10^6,

2-3x10⁵ and 1x10⁶ respectively.

DETD . . . baseline. Bound NS1 was eluted in 100 mM glycine, pH 2.5 with immediate neutralization by adding 1.5-2 ml of 1 **M** phosphate pH 7.4. Product purity was estimated at .about.95% by SDS-PAGE, while spectrometric methods indicated that the yield was roughly. . .

DETD . . . equivalent MAb could be prepared by those skilled in the art. Briefly, DEN-2 NS1 is purified from virally-infected mosquito cells (**e**.g. $C_{6/36}$ Aedes albopictis) using the procedure of Feighny et al ('92). The purified product is combined with Freund's adjuvant and. . . to standard procedures (Oi, V. T., L. A. Herzenberg. In Selected Methods in Cellular Immunology Mishell, B. B.; Shiigi, S. **M**., Eds; W. H. R. Freeman: San Francisco, 1980, Chapter 17). The fusion products are distributed in 96-well microtiter plates and. . .

DETD NS1 Augments Immunoprophylactic Activity of 80%**E** in Mice

DETD The protective capacity of a vaccine combining DEN-2 NS1 and 80%**E** immunogens was compared to one of identical composition except that NS1 was omitted. 80%**E** is a C-terminally truncated recombinant fragment of the DEN-2 **envelope** protein, expressed in Drosophila cells (Coller et al, submitted to Vaccine). Both immunogens were purified by immunoaffinity chromatography facilitated by MAbs 9D12 and 7E11, which are specific for the **dengue envelope** and NS1 proteins (see Example 6), respectively. Three week old BALB/c (HSD) mice were immunized via the subcutaneous route with adjuvanted preparations of 80%**E** and 80%**E**+NS1, as well as negative and positive control groups receiving saline (subcutaneous) and live DEN-2 virus injected intraperitoneally, respectively. For the experimental groups, five μg of each antigen (i.**e**. 5 μg 80%**E** and 5 μg 80%**E**+5 μg NS1) and one fg IscoMatrix.TM. (IscoTech) adjuvant was used per mouse/injection; primary immunizations were followed a week later by. . .

DETD . . . elicited complete protection, while 9/10 mice mock-immunized with saline were dead within two weeks of the viral challenge. Immunization with 80%**E**+NS1 also provided complete protection, while 80%**E** alone prevented death in 8 of 10 animals. In a separate experiment, 5 μg of IAC-purified NS1 plus1 μg IscoMatrix.TM.. . .

DETD The enhanced immunoprophylactic activity of 80%**E** combined with NS1 does not appear to be adjuvant-dependent as similar results were obtained using Freund's adjuvant rather than IscoMatrix.TM... . . tris/4 mM succinate pH 8.1, bound to DEAE-fractogel and eluted with a linear gradient of increasing NaCl concentration). As above, 80%**E** for this experiment was purified (.about.95% of total protein was 80%**E**) by immunoaffinity chromatography on a column of immobilized 9D 12 MAb. Three week old BALB/c mice were immunized with 25 μg of each antigen (i.**e**. 25 μg 80%**E** and 25 μg 80%**E**+25 μg NS1; weights refer to total amount of protein from each antigen preparation) administered intraperitonially in Freund's complete adjuvant. Booster. . .

DETD . . . completely protected, while 9 of 10 negative control animals receiving only saline injections succumbed to the viral challenge. Immunization with 80%**E** conferred protection to 5 of the 10 animals tested, while the surviving fraction was increased to 7 of 10 when. . . NS1 in a different adjuvant system, it is worth noting that we consistently obtain better immune responses to our recombinant **dengue** antigens using IscoMatrix.TM. instead of Freund's adjuvant.

. . . induces an immunological response in a host subject inoculated with said composition comprising a carrier and a mixture comprising a **Flavivirus** truncated **envelope** (**E**) protein and a **Flavivirus** nonstructural (NS) protein, wherein said nonstructural protein (NS) protein has been secreted as a recombinantly produced protein, from Drosophila cells, and wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

2. The immunogenic composition of claim 1, wherein said **envelope** protein (**E**) protein has been secreted as a recombinantly produced

protein from Drosophila cells.

4. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.

. . . Drosophila cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said nonstructural (NS) protein of the **Flavivirus** strain against which enhanced protection is sought; (b) recovering the nonstructural (NS) protein from the culture medium; and (c) combining said NS with a **Flavivirus** truncated **envelope** protein, wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

. . . Drosophila cells modified to contain a DNA molecule which comprises a nucleotide sequence encoding a nonstructural (NS1) protein of the **Flavivirus** against which enhanced protection is sought in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said NS1 protein of the **Flavivirus** strain against which enhanced protection is sought; (b) recovering the NS1 protein from the culture medium; and (c) combining said NS1 with a **Flavivirus** truncated **envelope** protein, wherein the truncated **envelope** (**E**) protein comprises approximately 80%**E**, wherein said 80%**E** represents a portion of the **envelope** protein that comprises approximately 80% of its length starting from amino acid 1 at its N-terminus.

10. An immunodiagnostic for the detection of a **Flavivirus**, wherein said immunodiagnostic comprises, the immunogenic composition of claim 1.

12. The immunodiagnostic of claim 10, wherein said **Flavivirus** is a **dengue** virus.

13. The immunodiagnostic of claim 10, wherein said **envelope** (**E**) protein has been secreted as a recombinantly produced protein from Drosophila cells.

L14 ANSWER 4 OF 15 USPATFULL on STN
2001:14256 Two-step immunization procedure against the pyramyxoviridae family of viruses using recombinant virus and subunit protein preparation.
Klein, Michel H., Willowdale, Canada
Tartaglia, James, Schenectady, NY, United States
Cates, George A., Richmond Hill, Canada
Ewasyshyn, Mary E., Willowdale, Canada
Virogeneitics Corporation, Troy, NY, United States (U.S. corporation)Connaught Laboratories Limited, North York, Canada (non-U.S. corporation)
US 6180398 B1 20010130
APPLICATION: US 1996-679065 19960712 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB An immunization strategy to provide protection against disease caused by infection with a paramyxoviridae virus, specifically respiratory syncytial virus (RSV) and parainfluenza virus, is described. A priming intranasal administration of a recombinant virus expressing at least one RSV or PIV protein or immunogenic sequence there first is made to the host followed by a booster administration of at least one purified RSV or PIV protein or immunogenic fragment thereof, which may be adjuvanted with alum. This immunization strategy provides a safe and effective means of controlling RSV and PIV infections. The strategy leads to a stronger protective immune response than other strategies and to the

induction of a more balanced Th 1/Th 2 type response than previously attained. Novel recombinant poxviruses are provided containing nucleic acid encoding a paramyxovirus protein or immunogenic fragment thereof is a non-essential region of the poxvirus genome, specifically NYVAC-F and ALVAC-F, which produce the F glycoprotein of RSV.

CLM What is claimed is:

1. A method of inducing an immune response in a host against disease caused by respiratory syncytial virus (RSV), which comprises: initially administering to the host an immunoeffective amount of a recombinant virus vector expressing at least one RSV protein or immunogenic fragment thereof; and subsequently administering to the host an immunoeffective amount of at least one purified RSV protein or immunogenic fragment thereof to achieve a RSV specific immune response in the host.

2. The method of claim 1 wherein said immune response in the host includes the production of virus specific neutralizing antibodies and/or virus specific cytotoxic T-cell responses.

3. The method of claim 2 wherein said recombinant virus is a recombinant pox virus.

4. The method of claim 2 wherein said recombinant virus expresses at least one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (**M**) proteins.

5. The method of claim 2 wherein said at least one purified RSV protein or immunogenic fragment thereof is selected from the group consisting of the fusion (F), attachment (G) and matrix (**M**) proteins.

6. The method of claim 5 wherein said recombinant virus is a recombinant pox virus.

7. The method of claim 5 wherein said recombinant virus expresses at least one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (**M**) proteins.

8. The method of claim 1 wherein the at least one purified RSV protein or immunogenic fragment thereof is administered with an adjuvant.

9. The method of claim 8 wherein the adjuvant is alum.

AI US 1996-679065 19960712 (8) <--

SUMM . . . a member of the Paramyxoviridae family of pneumovirus genus (ref. 2). The two major protective antigens of RSV are the **envelope** fusion (F) and attachment (G) glycoproteins (ref. 9).

SUMM . . . generated by the F and G glycoproteins, human cytotoxic T-cells have been shown to recognize the F protein RSV matrix (**M**) protein, nucleoprotein (N), small hydrophobic protein (SH) and nonstructural protein (1b) (ref. 10), produced following RSV infection. For PIV-3, the. . .

SUMM . . . of the virus to sialic acid containing host cell receptors. The type I F glycoprotein mediates fusion of the viral **envelope** with the cell **membrane** as well as cell to cell spread of the virus. It has recently been demonstrated that both the HN and F glycoproteins are required for **membrane** fusion. The F glycoprotein is synthesized as an inactive precursor (F) which is proteolytically cleaved into disulfide-linked F2 and F1. . .

SUMM The recombinant virus may express at least one of the F, G and **M** proteins of RSV or an immunogenic fragment thereof, particularly the F glycoprotein of RSV.

SUMM . . . thereof employed in the second or booster administration may be selected from the group consisting of the F, G, and **M** proteins of RSV or immunogenic fragments thereof and may compris a mixture of two or three of these RSV proteins. . .

SUMM The at least one purified paramyxoviridae protein or immunogenic fragment thereof, including mixtures of the F, G and/or **M** proteins or RSV or immunogenic fragments thereof, may be administered with an adjuvant or immunomodulator including alum.

DETD . . . one aspect, the at least one protein may comprise a mixture of RSV proteins such as G glycoprotein, F glycoprotein, **M** protein and heterodimers and oligomeric forms of the G and G proteins. Thus, the subsequent administration may be of a. . .

DETD G glycoprotein (95 kDa form) 10%
F_1 glycoprotein (48 kDa) 30%
M protein (31 kDa) 23%
F_2 glycoprotein (23 kDa) 19%

DETD . . . 6) (5'-GGTACTTGGAAGCTTTCAGTTACTAAATGCAAT-3') primed plasmid template pSKF7 for PCR. The 5' base pairs of RW356 contains the poxvirus early transcription termination **signal sequence** TTTTTGT, which has been inactivated by alternation to TTCCTGT, followed by a sequence which primes toward the RSV F gene. . .

DETD . . . was drained and filled again with 120 L of E199. The RSV inoculum was added at a multiplicity of infection (**M**.O.I.) of 0.001 and the culture was then maintained for 3 days before one-third to one-half of the media was drained. . . stirring was stopped and the beads allowed to settle. The viral culture fluid was drained and filtered through a 20 μm filter followed by a 3 μm filter prior to further processing. The clarified viral harvest was concentrated 75-100 fold using tangential flow ultrafiltration with 300 NMWL **membrane** and diafiltered with phosphate buffered saline containing 10% glycerol. The viral concentrate was stored frozen at -70° C. prior to. . . rotor at 4° C. In some instances the viral pellet was suspended in 1 mM sodium phosphate, pH 6.8, 2 **M** urea, 0.15 **M** NaCl, stirred for 1 hour at room temperature, and then recentrifuged at 15,000 RPM for 30 min. in a Sorvall. . .

DETD . . . mouse monoclonal antibody (mAb 131-2G), to G glycoprotein (FIGS. 2b and 3b) or guinea pig anti-serum (gp178) against an RSV **M** peptide (peptide sequence: LKSKNMLTTVKDLTMKTLNPTHDIIALCEFEN-SEQ ID No: 20) (FIGS. 2c and 3c), or goat anti-serum (Virostat #0605) against whole RSV (FIGS.. . .

DETD **M** protein (31 kDa)=23%

DETD . . . washing buffer, and hydrogen peroxide (substrate) in the presence of tetramethylbenzidine was added. The reaction was stopped by adding 2 **M** sulfuric acid. The colour was read in a Multiscan Titertek plate reader at an optical density (OD) of 450 nm.. . .

DETD 1. Glezen, W. P., Paredes, A. Allison, J. **E**., Taber, L. H. and Frank, A. L. (1981). J. Pediatr. 98, 708-715.

DETD 2. Chanock, R. **M**., Parrot, R. H., Connors, **M**., Collins, P. L. and Murphy, B. R. (1992) Pediatrics 90, 137-142.

DETD 7. Wertz, G. W., Sullender, W. **M**. (1992) Biotech. 20, 151-176.

DETD 8. Fulginiti, V. A., Eller, J. J., Sieber, O. F., Joyner, i. W., Minamitani, **M**. and Meiklejohn, G. (1969) Am. J. Epidemiol. 89 (4), 435-448.

DETD 9. McIntosh, K. and Chanock, R. **M**. (1990) in Fields Virology (Fields, B. **M**., and Knipe D. **M**. eds.) pp. 1045-1075, Raven Press, Ltd., New York.

DETD 11. Chin, J., Magoffin, R. L., Shearer, L. A., Schieble, J. H. and Lennette, **E**. H. (1969) Am. J. Epidemiol. 89 (4), 449-463.

DETD 12. Kapikian, A. Z., Mitchell, R. H., Chanock, R. **M**., Shvedoff, R. A. and Stewart, C. **E**. (1969) Am. J. Epidemiol. 89 (4), 405-421.

DETD 13. Connors, **M**., Collins, P. L., Firestone, C. Y., Sotnikov, A. V., Waitze, A., Davis, A. R., Hung, P. P., Chanock, R. **M**., Murphy, b. (1992) Vaccine, 10, 475-484.

DETD 14. Walsh, **E**. **E**., Hall, C. B., Briselli, **M**., Brandiss, **M**. W. and Schlesinger, J. J. (1987) J. Infect. Dis. 155 (6), 1198-1204.

DETD 15. Connors **M**, Kulkarni, CY, Firestone KL, Holmes KL, Morse III HC, Sotnikov AV, and Murphy BR, (1992). J. Virol. 66: 7444-7451.

DETD 16. Connors **M**, Giese NA, Kulkarni AB, Firestone CY, Morse III HC, and Murphy BR, (1994) J. Virol. 68: 5321-5325.

DETD 19. Tartaglia, J., Perkus, **M**. **E**., Taylor, J., Norton, **E**. K., audonnet, J. -C., Cox, W. I., Davis, S. W., VanderHoeven, J., Meignier,

B., Riviere, M., Languet, B., Paoletti, E. (1992) NYVAC: a highly attenuated strain of vaccinia virus. Virology 188, 217-232.
DETD 20. Konishi, E., Pincus, S., Paoletti, E., Laegried, W. W., Shope, R. E., Mason, P. W. (1992) A highly attenuated host-range restricted vaccinia virus strain, NYVAC, encoding the **prM**, **E**, and NS1 genes of Japanese **encephalitis virus** prevents JEV viremia in swine. Virology 190, 454-458.
DETD 21. Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R., Paoletti, E. (1988a) Protective immunity against avian influenza induced b a fowlpoxvirus recombinant. Vaccine 6, 504-508.
DETD 22. Taylor, J., Weinberg, R., Languet, B., Desmettre, Ph., Paoletti, E. (1988b) Recombinant fowlpoxvirus inducing protective immunity in non-avian species. Vaccine 6, 497-503.
DETD 23. Taylor, J., Trimarchi, C., Weinberg, R., Languet, B., Guillemin, F., Desmettre, Ph., Paoletti, E. (1991) Efficacy studies on a canarypox-rabies recombinant. Vaccine 9, 190-193.
DETD 24. Taylor, J., Weinberg, R., Tartaglia, J., Richardson. C., Alkatib, G., Briedis, D., Appel, M., Norton, E., Paoletti, E. (1992) Nonreplicating viral vectors as potential vaccines: recombinant canarypox virus expressing measles virus fusion (F) and hemagglutinin (HA) glycoproteins. Virology. . .
DETD 25. Tartaglia, J., Taylor, J., Cox, W. I., Andonnet, J. -C., Perkus, M. E., Raedelli, A., de Giuli Morghen, C., Meignier, B., Riviere, M., Weinhold, K., Paoletti, E. (1993) Novel poxvirus strains as research tools and vaccine vectors. In AIDS Research Reviews (W. C. Koff, F. Wong-Staal, and. . .
DETD 26. Cadoz, M., Strady, A., Meignier, B., Taylor, J., Taryaglia, J., Paoletti, E., Plotkin, S. (1992) Immunization with canarypox virus expressing rabies glycoproteins, Lancet 339, 1429-1432.
DETD . . . Excler, J. -L., Riviere, Y., Gonzalez-Canali, G., Feuillia, V., Coulaud, P., Gluckman, J. -C., Matthews, T. J., Meignier, B., Kieny, M. -P., Gonnet, P., Diaz, I., Meric, C., Paoletti, E., Tartaglia, J., Salomon, H., Plotkin, S. (1995) A primeboost approach to HIV preventive vaccine using a recombinant canarypox virus expressing. . .
DETD 29. Perkus, M., Limbach, K. and Paoletti, E. (1989) Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. J. Virol. 63: 3829-3836.
DETD 31. Goebel, S., Johnson, G., Perkus, M., Davis, S., Winslow, J., and Paoletti, E. (1990), The complete DNA sequence of vaccinia virus, Virology 179: 247-266.
DETD 33. Fulginiti, V. A., Eller, J. J., Sieber, O. F., Joyner, J. W., Minamitani, M. and Meiklejohn, G. (1969) Am. J. Epidemiol. 89 (4), 435-448.
DETD 34. Chin, J., Magoffin, R. L., Shearer, L. A., Schieble, J. H. and Lennette, E. H. (1969) Am J. Epidemiol. 89 (4), 449-463.
DETD 35. Jensen, K. E., Peeler, B. E. and Dulworth, W. G. (1962) J. Immunol. 89, 216-226.
DETD 36. Murphy, B. R., Prince, G. A., Collins, P. L., Van Wyke Coelingh, K., Olmsted, R. A., Spriggs, M. K., Parrott, R. H., Kim, H. -Y., Brandt, C. D. and Chanock, R. M. (1988) Vir. Res. 11, 1-15.
DETD 37. Hall, S. L., Sarris, C. M., Tierney, E. L., London, W. T., and Murphy, B. R. (1993) J. Infect. Dis. 167, 958-962.
DETD Belshe, R. B., Karron, R. A., Newman, F. K., Anderson, E. L., Nugent, S. L., Steinhoff, M., Clements, M. L., Wilson, M. H., Hall, S. L., Tierney, E. L. and Murphy B. R. (1992) J. Clin. Microbiol. 30 (8), 2064-2070.
DETD 39. Hall, S. L., Stokes, A., Tierney, E. L., London, W. T., Balshe, R. B., Newman, F. C. and Murphy, B. R. (1992) Vir. Res. 22, 173-184.
DETD 40. Van Wyke Coelingh, K. L., Winter, C. C., Tierney, E. L., London, W. T. and Murphy, B. R. (1988) J. Infect. Dis. 157 (4), 655-662.
DETD 41. Ray, R., Novak, M., Duncan, J. D., Matsuoka, Y. and Compans, R. W. (1993) J. Infect. Dis. 167, 752-755.
DETD 42. Ray, R., Brown, V. E. and Compans, R. W. (1985) J. Infect. Dis. 152 (6), 1219-1230.
DETD 47. Ewasyshyn, M., Caplan, B., Bonneau A. -M., Scollard, N., Graham, S., Usman, S. and Klein, M. (1992) Vaccine 10 (6), 412-420.

DETD 48. Ambrose, M. W., Wyde, P. R., Ewasyshyn, M., Boileau, A. M., Caplan, B., Meyer, H. L. and Klein, M. (1991) Vaccine 9, 505-511.

DETD 50. Lehman, D. J., Roof, L. L., Brideau, R. J., Aeed, P. A., Thomsen, D. R., Elhammer, A. P., Wathen, M. W. and Homa, F. L. (1993) J. Gen. Virol. 74, 459-469.

DETD 51. Brideau, R. J., Oien, N. L., Lehman, D. J., Homa, F. L. and Wathen, M. W. (1993) J. Gen. Virol. 74, 471-477.

. . . one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

. . . RSV protein or immunogenic fragment thereof is selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

. . . one RSV protein or immunogenic fragment thereof selected from the group consisting of the fusion (F), attachment (G) and matrix (M) proteins.

L14 ANSWER 5 OF 15 USPATFULL on STN

2000:174106 Subunit immonogenic composition against **dengue** infection.

Ivy, John, Kailua, HI, United States
Nakano, Eilen, Hon., HI, United States
Clements, David, Honolulu, HI, United States
Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)

US 6165477 20001226

APPLICATION: US 1997-915152 19970820 (8) <--

DOCUMENT TYPE: Utility; Granted.

CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, Japanese encephalitis (JE) virus, tick-borne **encephalitis virus** (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), **membrane** (M), and **envelope** (E) proteins. The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the **E** glycoprotein that retain their native configuration and attendant properties associated therewith (i.e., ability to induce neutralizing antibody responses). To date, recombinantly produced **E** glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the **E** glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of **E** containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in Drosophila melanogaster Schneider cell lines. Immunogenic compositions comprising these recombinant **envelope** glycoproteins were capable of inducing protective, neutralizing antibody responses when administered to a suitable host.

CLM What is claimed is:

1. An immunogenic composition which generates protective, neutralizing antibody responses to a **Flavivirus** in a murine host which responses confer protection against intracerebral challenge by the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of Japanese **encephalitis virus** (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne **encephalitis virus** (TBE) which composition contains an adjuvant; and a portion of the **envelope** protein (**E**) of the

Flavivirus strain against which said responses are sought, which portion is 80% **E**, wherein said 80% **E** represents that portion of the **envelope** protein that constitutes 80% of its length starting from amino acid 1 at its N-terminus and which portion has been secreted as a recombinantly produced protein from Drosophila cells.

2. The immunogenic composition of claim 1 wherein said Drosophila cells are D. melanogaster Schneider cells.

3. The immunogenic composition of claim 1 wherein said adjuvant is an alum adjuvant.

4. An immunogenic composition which generates a neutralizing antibody response to a **Flavivirus** in a murine host against the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of Japanese **encephalitis virus** (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne **encephalitis virus** (TBE) which composition contains an adjuvant; and a portion of the **envelope** protein (**E**) of the **Flavivirus** strain against which generation of said response is sought, which portion is 80% **E**, wherein said 80% **E** represents that portion of the **envelope** protein that constitutes 80% of its length starting from amino acid 1 at its N-terminus, and which portion has been secreted as a recombinantly produced protein from Drosophila cells.

5. The immunogenic composition of claim 4 wherein said Drosophila cells are Schneider cells.

6. The immunogenic composition of claim 4 wherein said **Flavivirus** is a **dengue** virus.

7. The immunogenic composition of claim 4 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA_L).

8. The immunogenic composition of claim 4 wherein the adjuvant is an alum adjuvant.

9. A method to generate a neutralizing antibody response in a non-human subject against a **Flavivirus** strain, said strain selected from the group consisting of a strain of **dengue**, a strain of YF, a strain of JEV, and a strain of TBE, which method comprises administering to a non-human subject in need of generating said response an effective amount of the immunogenic composition of claim 4.

10. The method of claim 9 wherein said **Flavivirus** is a **dengue** virus.

11. The immunogenic composition of claim 1 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from a human tissue plasminogen activator prepropeptide secretion leader (tPA_L) sequence.

12. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.

13. The immunogenic composition of claim 2 wherein the Flaviririus is a **dengue** virus.

TI Subunit immonogenic composition against **dengue** infection

AI US 1997-915152 19970820 (8) <--

AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, Japanese encephalitis (JE) virus, tick-borne **encephalitis virus** (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell

membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), **membrane** (M), and **envelope** (E) proteins. The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the **E** glycoprotein that retain their native configuration and attendant properties associated therewith (i.**e**., ability to induce neutralizing antibody responses). To date, recombinantly produced **E** glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the **E** glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of **E** containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in Drosophila melanogaster Schneider cell lines. Immunogenic compositions comprising these recombinant **envelope** glycoproteins were capable of inducing protective, neutralizing antibody responses when administered to a suitable host.

SUMM The invention relates to protection against and diagnosis of **dengue** fever. More specifically, the invention concerns a subunit of the **dengue** virus **envelope** protein secreted as a mature recombinantly produced protein from eucaryotic cells which is protective against **dengue** infection, which raises antibodies useful in passive immunization, and which is useful in diagnosis of infection by the virus.

SUMM The **dengue** viruses are members of the family **Flaviviridae** which also includes the Japanese encephalitis (JE) virus, Tick-borne encephalitis (TBE) virus, and the initially discovered prototype of this class, the yellow fever (YF) virus. The **flaviviruses** contain a single positive strand genomic RNA and are small enveloped viruses affecting animals, but generally transmitted to vertebrates by chronically infected mosquito or tick vectors. **Flaviviruses** are enveloped by host cell **membrane** and contain the three structural proteins capsid (C), **membrane** (M), and **envelope** (E). The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated, whereas **M** is not, although its precursor, **prM**, is a glycoprotein. Glycoprotein **E**, the largest structural protein, contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. It is also a. . .

SUMM **Dengue** virus is the causative agent of **dengue** fever and is transmitted to man by Aedes mosquitoes, principally Aedes aegypti and Aedes albopictus. Classic **dengue** fever is an acute illness marked by fever, headache, aching muscles and joints, and rash. A fraction of cases, typically in children, results in more extreme forms of infection, i.**e**., **dengue** hemorrhagic fever (DHF) or **dengue** shock syndrome (DSS). Without diagnosis and prompt medical intervention, the sudden onset and rapid progress of DHF/DSS can be fatal.

SUMM **Dengue** is one of the most important virus groups transmitted to man by arthropods in terms of global morbidity; it has been estimated that **dengue** is responsible for up to 100 million illnesses annually. With the advent of modern jet travel, **dengue** has spread globally in the tropics and subtropics, and multiple **dengue** serotypes in a region are common.

SUMM Every **flavivirus** genome is a single positive-stranded RNA of approximately 10,500 nucleotides containing short 5' and 3' untranslated regions, a single long. . . products encoded by the single, long open reading frame are contained in a polyprotein organized in the order, C (capsid), **prM/M** (**membrane**), **E** (**envelope**), NS1 (nonstructural), NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Chambers, T. J. et al. Ann Rev Microbiol (1990) 44:649-688). Processing. . . comparing the nucleotide sequence and the amino terminal sequences of the viral proteins. Subsequent to initial processing of the polyprotein, **prM** is converted to **M** during virus release (Wengler, G. et al. J Virol (1989)

65:2521-2528), and anchored C is processed during virus maturation (Nowak. . .

SUMM There are four antigenically related **dengue** viruses which, however, can be recognized as distinct serotypes. The complete genomic sequence for at least one strain of each of the four **dengue** serotypes has been reported (DEN-1, Fu, J. et al. Virology (1992) 188:953-958; DEN-2, Deubel, V. et al. Virology (1986) 155:365-377;. . . 162:167-180; DEN-3, Osatomi, K. et al. Virus Genes (1988) 2:99-108; Osatomi, K. et al. Virology (1990) 176:643-647; DEN-4, Zhao, B. **E**. et al. Virology (1986) 155:77-88; Mackow, **E**. et al. Virology (1987) 159:217-228). In addition, the compete genomic sequences of other **flaviviruses** are known (**e**.g., YF virus: Rice et al., Science (1985) 229:726-733).

SUMM It does not appear that infection by one **dengue** serotype can confer long-term immunity on the individual with respect to other serotypes. In fact, secondary infections with heterologous serotypes. . . antibodies are directed against type-specific determinants. On the other hand, secondary infections by heterologous serotypes generate IgG antibodies which are **flavivirus** crossreactive.

SUMM Helpful reviews of the nature of the **dengue** disease, the history of attempts to develop suitable vaccines, and structural features of **flaviviruses** in general as well as the molecular structural features of the **envelope** protein of **flaviviruses** are found in Halstead, S. B. Science (1988) 239:476-481; Brandt, W. **E**. J Infect Disease (1990) 162:577-583; Chambers, T. J. et al. Annual Rev Microbiol (1990) 44:649-688; Mandl, C. W. et al. Virology (1989) 63:564-571; and Henchal, **E**. A. and J. R. Putnak, Clin Microbiol Rev (1990) 3:376-396.

SUMM A successful vaccine for protection against **dengue** infection has never been developed. However, there have been a number of preliminary efforts, many of which focus on the **envelope** protein, since this protein is exposed at the surface and is believed to be responsible for eliciting immunity.

SUMM Monoclonal antibodies (Mabs) directed against purified **E** of several **flaviviruses** DEN-2 (Henchal et al. Am J Trop Med Hyg (1985) 34:162-169, TBE (Heinz, F. X. et al. Virology (1983) 126:525-537),. . .

SUMM Although the primary amino acid sequence of the **flavivirus E** glycoprotein is variable (45-80% identity), all have twelve conserved cysteine residues, forming six disulfide bridges, and hydrophilicity profiles are nearly. . . antibody competitive binding studies, monoclonal antibody binding to purified proteolytic fragments, and analysis of neutralizing antibody escape mutants of Tick-Borne **Encephalitis Virus**, glycoprotein **E** was divided into three antigenic domains (A, B, and C) and two transmembrane segments at its carboxy-terminus. See, for example,. . .

SUMM . . . 200-250 containing five of the six disulfide bridges. Neutralization and hemagglutination inhibition epitopes are found within domain A, and, for **dengue** viruses, one of the two N-linked glycosylation sites. A conserved hydrophobic amino acid sequence within domain A as been postulated to provide fusogenic properties after low pH treatment. Amino acid sequences conserved among the **flavivirus** family are located within this region; thus, broadly **flavivirus**-cross-reactive epitopes lie within this domain.

SUMM . . . was identified as a continuous domain composed of amino acids 301-395 (an approximate region between amino acids 300-400 for all **flaviviruses**). The domain can be isolated as a single immunoreactive proteolytic fragment. It has been postulated that this domain forms part. . .

SUMM Many strategies are currently under investigation to develop an effective and safe **dengue** vaccine; however, to date, no single strategy has proven completely satisfactory. Attempts to generate live attenuated **dengue** vaccine strains have not been entirely successful, although research into this area continues. In the absence of effective, live attenuated **dengue** vaccines, a significant effort has been invested in the development of recombinant, **dengue** subunit or viral-vectored vaccines.

SUMM Recombinant **dengue** proteins have been expressed in several systems to

date (see Putnak, R. A. (1994) Modern Vaccinology, E. Kurstak ed., Plenum Medical, New York, pp. 231-252, for review). Most efforts using Escherichia coli have yielded poor immunogen unable to elicit neutralizing antibodies. This may reflect non-native conformation of **dengue** proteins expressed in the bacteria and the necessity to process the viral proteins through the secretion pathway in order to. . .

SUMM Several reports have described vaccinia-**flavivirus** recombinants expressing **envelope** protein as part of a polyprotein (**e**.g. C-**prM**-**E**-NS1; [**Dengue**] Zhao, B. G. et al. J Virol (1987) 61:4019-4022; Deubel, V. et al. J Gen Virol (1988) 69:1921-1929; Bray, **M**. et al. J Virol (1991) 63:2853-2856; [YF] Hahn, Y. S. et al. Arch Virol (1990) 115:251-265), as a single protein (**e**.g. 100% **E**; [**Dengue**] Bray, **M**. et al., J Virol (1989) 63:2853-2856), or as polypeptides (**e**.g. 79% **E**-RKG; Men, R. et al. J Virol (1991) 65:1400-1407). The most successful recombinant vaccinia viruses, those capable of inducing neutralizing antibodies and protecting mice from virus challenge, were those which were secreted **E** extracellularly or accumulated **E** on the cell surface.

SUMM Men, R. et al. (1991, supra) described the recombinant production of various C-terminal truncations of the DEN-4 **envelope** protein using a recombinant Vaccinia virus vector and infecting mammalian CVI cells. The results showed that the recombinants that contain greater than 79% of the coding sequence produced an intracellular protein that could be immunoprecipitated with anti-**dengue** virus antibodies contained in hyperimmune mouse ascitic fluid (HMAF). Although there was a reduced level of detection for protein which contained 79% of **envelope** or less, this did not appear to result from reduced production of the protein. It was also found that only truncations which contained 79% of **E** or less were secreted efficiently; **E** polypeptides equal to or larger than 81% **E** were not secreted efficiently.

SUMM Men et al. (1991, supra) constructed additional C-terminal truncations between 79% **E** and 81% **E** to map the amino acids responsible for the difference in secretion and immunoreactivity with HMAF of these two truncated **E** polypeptides. The results demonstrated that 79% **E** containing the additional tripeptide sequence RKG was also secreted. Although both 59% **E** and 79% **E**-RKG were secreted, only 79% **E**-RKG was detected at the cells' surface. The recombinant Vaccinia viruses containing various truncations were also used to immunize mice. Mice immunized with recombinants expressing 79% **E**-RKG or larger portions of the **envelope** protein were protected. However, except for 59% **E**, mice immunized with 79% **E** or a smaller product were only partially protected. The 59% **E** elicited high protection rates (>90%) comparable to 79% **E**-RKG and larger C-terminal truncated **E** polypeptides. Protection correlated with binding to HMAF.

SUMM . . . Putnak, R. A. et al. Am J Trop Med Hyg (1991) 45:159-167; Deubel, V. et al. Virology (1991) 180:442-447). Baculovirus-expressed **dengue** and JE **E** glycoprotein elicited neutralizing antibodies, protected mice from a lethal **dengue** virus challenge, or both. In spite of these successes, the expression levels reported in baculovirus are low and the recombinant. . .

SUMM Research with purified polypeptides released by proteolysis of **flavivirus envelope** proteins, with recombinant polypeptides, and with synthetic peptides has indicated where protective epitopes may map. The isolated 9000 dalton domain. . .

SUMM A cyanogen bromide-cleaved 8 kD fragment (amino acids 375-456) overlapping domain B from JE **envelope** protein was found to induce neutralizing antibodies in mice (Srivastava, A. K. et al. Acta Virol (1990) 34:228-238). Immunization of mice with a larger polypeptide (JE **E** amino acid 319 to NS1 amino acid 65) spanning the 8 kD peptide expressed in Escherichia coli as a fusion. . .

SUMM Mason, P. W. et al. J Gen Virol (1990) 71:2107-2114 identified two domains of the DEN-1 **envelope** protein: domain I which includes amino acids 76-93 of the **E** protein and domain II (equivalent to domain B) which includes amino acids 293-402. These domains were identified from deletion analysis using recombinant fusion proteins expressed in **E**. coli and reacted with antiviral monoclonal antibodies. Recombinant

fusion proteins containing E. coli trpE sequences fused to the **envelope** protein (amino acids 1 to 412) elicited antibodies in mice which reacted with a portion of the protein containing domain. . .

SUMM In addition, Mason, P. W. et al. (J Gen Virol (1989) 70:2037-2049) expressed a collection of **E**. coli trpE fusion proteins to segments of JE virus **envelope** protein spanning domain B. The trpE fusion proteins containing the smallest JE **E** fragments that retained immunoreactivity with a panel of neutralizing monoclonal antibodies included amino acid residues from methionine 303 through tryptophan. . .

SUMM Trirawatanapong, T. et al. Gene (1992) 116:139-150 prepared several truncated forms of **dengue** 2 **envelope** proteins in **E**. coli for epitope mapping, and mapped monoclonal antibody 3H5 to its corresponding epitope. This was first localized between amino acids. . . amino acids between positions 386 and 397. The mapping was apparently confirmed by the ability of a synthetic peptide containing **E** protein amino acids 386-397 to bind 3H5 specifically.

SUMM Megret, F. et al. Virology (1992) 187:480-491 prepared 16 overlapping fragments of DEN-2 **envelope** protein as trpE fusion products in **E**. coli for epitope mapping. The fusion proteins are produced intracellularly and obtained from the lysates. These products were used to. . .

SUMM . . . findings of Trirawatanapong et al. Gene (1992, supra), MAb 3H5 was unable to bind to trpE fusion proteins containing DEN-2 **E** amino acids 304-397, 298-385, or 366-424. The two exceptional MAbs in the findings of Megret et al. are MAbs 5A2. . .

SUMM Although it appears established from the art that the B domain of the **flavivirus envelope** protein contains epitopes which bind neutralizing antibodies, problems have arisen with respect to producing recombinant polypeptides containing the B domain. . . a form which mimics the native protein and is thus capable of eliciting an immune response. The only recombinantly produced **E** polypeptides containing the B domain that elicited a protective immune response in mice were expressed from Vaccinia and baculovirus vectors.. . .

SUMM It has now been found that the B domain of the **envelope** protein can be successfully secreted from yeast in a form which elicits the production of neutralizing antibodies. This permits, for the first time, the production of a successful recombinantly produced subunit **dengue** vaccine.

SUMM The invention provides vaccines containing, as an active ingredient, a secreted recombinantly produced the **dengue envelope** protein or a subunit thereof. The vaccines are capable of eliciting the production of neutralizing antibodies against **dengue** virus. In the illustrations below, the B domain of the **envelope** protein (**E**) is secreted from yeast by producing it in an expression vector containing the α-mating factor prepropeptide leader sequence (preproMFα_L). Peptide subunits representing 60% **E** and 80% **E** are secreted from Drosophila cells using the human tissue plasminogen activator secretion **signal sequence** for the propeptide (tPA$_L$) or from the homologous **premembrane** (**prM**) leader. The secreted products can easily be purified and prepared as a vaccine.

SUMM Thus, in one aspect, the invention is directed to a vaccine for protection of a subject against infection by **dengue** virus. The vaccine contains, as active ingredient, the **envelope** protein of a **dengue** virus serotype or a subunit thereof. The **E** or subunit is secreted as a recombinantly produced protein from eucaryotic cells. The vaccine may further contain portions of additional **dengue** virus serotype **E** proteins similarly produced.

DRWD FIG. 1 is a drawing reproduced from Mandl, et al. (supra) showing a model of the **envelope** protein of **flaviviruses**.

DRWD FIG. 10 shows the survival times of mice immunized with recombinant domain B and challenged with **Dengue**-2.

DRWD FIG. 13 shows the survival times of mice immunized with D. melanogaster Schneider cell-secreted 80% **E**.

DETD . . . subunit vaccine that can be efficiently produced recombinantly and secreted and that is effective in protecting subjects against infection with **dengue** virus. Although many attempts have been made to

obtain such a subunit vaccine, either the subunit itself is resistant to. . . recombinant production is facile, it fails to elicit neutralizing antibodies. The present applicants have found that certain portions of the **envelope** protein of **dengue** virus type 2, such as domain B representing approximately 100 amino acids of the **envelope** protein extending approximately from the Gly at position 296 to the Gly at position 395, and optionally including additional **E** sequence through position 413 of the protein, and other portions of **E**, i.**e**., 60% **E** and 80% **E** are effectively secreted by certain convenient eucaryotic recombinant hosts, in a form that permits processing to mimic the native conformation. . . to raise neutralizing antibodies in animals. Thus, this subunit represents a useful component of a vaccine for protecting subjects against **dengue** infection.

DETD . . . used herein, "B domain" refers to a peptide which spans from approximately Gly 296 to Gly 395 of the DEN-2 **envelope** protein, and optionally including additional **E** sequence through position 413 of the **envelope** protein. These positions are approximate; for example, Mandl (1989, supra) describes the generation of a tryptic fragment containing domain B which spans the amino acids of the TBE **E** protein from position 301 to 396. The sequences described in the present application represent the **envelope** protein from **dengue** Type 2 virus (DEN-2); three additional distinct **dengue** serotypes have been recognized. Therefore, "Domain B" also refers to the corresponding peptide region of the **envelope** protein of these serotypes, and to any naturally occurring variants. In addition, B domain includes extended forms of the about. . . extensions do not interfere with the immunogenic effectiveness or secretion of the B domain. In one embodiment, such extensions are minimal--i.**e**., not more than six additional amino acids--at either the N-terminus or the C-terminus, or distributed between these termini; preferably no. . .

DETD . . . domain includes at least portions of the region extending to amino acid 413, the additional region may confer additional functions, **e**.g., enhancing immunogenicity by providing a helper T cell epitope. The form of domain B which includes positions about 296-413 is. . .

DETD Other portions of the **E** protein illustrated below are self-explanatory. 80% **E** is the N-terminal 80% of the protein from residue 1 to residue 395. 60% **E** represents the corresponding shorter sequence. These subunits are produced from vectors containing the DNA encoding the mature protein, or along with the **prM** fusion which results in secretion of the 80% or 60% **E** per se.

DETD . . . the subunits as produced must assume a conformation and undergo processing under conditions which render them similar to the native **envelope** portion as it exists in the **envelope** protein of the virus. In order to achieve this, the recombinant production must be conducted in eucaryotic cells, preferably yeast. . . ovary cells. Other insect cells may also be used in conjunction with baculovirus based vectors. The B domain or 60% **E** or 80% **E** must be produced as a correctly processed protein and secreted.

DETD . . . ways. First, this can be done by expressing the B domain in yeast in operable linkage with the α-mating factor **signal sequence**. Constructs which place the nucleotide sequence encoding the B domain disposed so as to encode a fusion protein with an upstream α-mating factor **signal sequence** are therefore included within the scope of the invention. An additional preferred embodiment employs Drosophila cells and the human tissue plasminogen activator leader sequence for secretion of 60% **E** or 80% **E** as well as domain B. **Envelope** protein subunits that represent N-terminal portions of truncated protein may also be secreted from the homologous **prM** fusion. Other secretion signal peptides or secretion leader pre/pro peptides, such as those associated with invertase or acid phosphatase of. . . In general, the invention includes expression systems that are operable in eucaryotic cells and which result in the formation of **envelope** protein or a subunit secreted into the medium. Thus, useful in the invention are cells and cell cultures which contain. . .

DETD The properly processed **E** protein or subunit is recovered from the cell culture medium, purified, and formulated into vaccines. Purification and

vaccine formulation employ. . .

DETD To immunize subjects against **dengue** fever, the vaccines containing the subunit are administered to the subject in conventional immunization protocols involving, usually, multiple administrations of. . .

DETD . . . can themselves be used as passive vaccines. For production of passive vaccine, a suitable mammalian subject is immunized with the **E** protein or subunit of the invention and antibodies are either recovered directly as a composition from the antisera or indirectly. . .

DETD In addition to use in vaccines or in the generation of passive vaccines, the mature recombinant **E** protein and subunits of the invention may be used as analytical reagents in assessing the presence or absence of antidengue antibodies in samples. The interest in doing this may be diagnosis of infection with **dengue**, monitoring response to **dengue** infection or may simply reside in the use of immunoassays as part of standard laboratory procedures in the study of. . .

DETD Thus, the secreted protein, such as 60% **E**, 80% **E** or B domain may be adsorbed onto solid support and the support then treated with a sample to be tested. . .

DETD In addition, both the mature peptides, such as domain B and 60% **E** or 80% **E** of the invention and the antibodies immunoreactive with it can be used in standard purification procedures as affinity reagents. Thus,. . .

DETD In the examples below, particular subunits of the **dengue** Type 2 **envelope** protein, in particular 60% **E**, 80% **E** and domain B are illustrated as representative of effective subunits of the **envelope** protein. For the 60% **E** and 80% **E** constructs in general, secretion can be obtained from constructions designed to express the prME subunit fusion. The mature N-terminus of the **envelope** protein is then secreted into the culture medium. Whether the N-terminus of the **envelope** protein subunits were fused to a heterologous leader, such as the human tissue plasminogen activator leader sequence, or to the homologous **prM** sequence, the mature form of the truncated **envelope** protein is secreted. The secreted truncated Es are expressed at high levels in Drosophila, efficiently processed, and secreted into the medium. The products are glycosylated and processed to an endo-H resistant form. The secreted form of truncated **E** produced cotranslationally with **prM** generally represents about 20-30% of the total protein in the medium. Furthermore, based upon reactivity with conformationally sensitive monoclonal antibodies, using a ELISA and immunofluorescence formats, the secreted **E** products are shown to have a native conformation. Immunization of mice with crude medium from transformed cells expressing **prM** truncated **E** induces a potent virus-neutralizing response.

DETD Preparation of **Envelope** Proteins in Saccharomyces cerevisiae

DETD A cDNA clone derived from **dengue** serotype 2 (DEN-2) described by Hahn, Y. S. et al. Virology (1988, supra) was used as the starting material. This. . .

DETD FIG. 2 shows the sequence of the cDNA derived from genomic RNA of DEN-2 PR159/S1 for the Capsid, **preMembrane**, **Envelope**, and NS1 genes. Shown in bold at nucleotides 103, 1940, 1991, and 2025 are corrections to the Hahn published sequence.. . . sequence from the wild-type sequence are noted above the wild-type sequence. There are no nucleotide differences in the Capsid and **preMembrane** protein-encoding portions and there are four in the **E** encoding portion.

DETD FIG. 3 shows the cDNA sequence of DEN-2 PR159/S1 for the Capsid, **preMembrane**, **Envelope**, and NS1 genes and the inferred translation of those four genes, which is part of the larger **dengue** polyprotein. The four differences between wild-type DEN-2 PR159 and the S1 strain are shown above the S1 nucleotide sequence. Also. . .

DETD In the **E** gene, three of the four mutations are silent; S1 has G instead of A at position 1314, T rather than. . . conservative, mutagenesis studies of other viral structural proteins (Coller, B. G. et al. (1994) Mutagenesis Studies on the Predicted VP2 **E**-F Loop of Coxsackievirus B3, Abstract, 13th Annual Meeting of the American Society for Virology) have demonstrated that even relatively conservative. . .

DETD Various **E** gene subclones were obtained which represented the amino-terminal 90% of the **envelope**, 80% of the **envelope**, 60% of the

envelope and classical domain B. Using the assignment of Mandl, C. W. et al. J Virol (1989) 63:564-571, classical domain B. . . . et al. at its carboxy end which can be included in some forms of the domain B of the invention, e.g., DomB+T.

DETD The portion of the genome that encodes 80% of the **envelope** protein (80% **E**) was amplified using the Polymerase Chain Reaction, primers D2E937p and D2E2121m, and plasmid pC8 (Hahn et al. (1988, supra) as. . .

DETD In this notation of the primers, the virus serotype is first indicated (D2 for DEN-2), then the corresponding **dengue** gene--i.e., in this case **envelope**, **E**, is noted. Then is noted the number in the **dengue** cloned sequences of FIGS. 2 or 3 for the first **dengue** nucleotide in the 5'-3' direction of the oligonucleotide, i.e., using the numbering of Hahn et al. (1988, supra), and finally the notation shows whether the oligonucleotide primes the plus (p) or the minus (m) strand synthesis. The sequence in the primers corresponding to **dengue** cDNA are written in uppercase letters; nondengue sequence is written in lowercase letters.

DETD The D2E2121m primer placed two stop codons after the 395th codon of **E**. The 80% **E** amplified cDNA fragment was digested at the XbaI sites in the cloning adapters and cloned into the NheI site of pBR322 to obtain p29D280E. Double-strand sequence for 80% **E** was determined, which identified a single silent PCR-introduced mutation at nucleotide 2001 (AAC/Asn to AAT/Asn).

DETD A subclone representing domain B was obtained from the 80% **E** subclone by oligonucleotide-directed mutagenesis. In the mutagenesis, stop codons and restriction endonuclease sites were inserted between domain C- and domain. . . FIG. 4, to avoid a high AT content in the mutagenic oligonucleotide, the stop codons defining the carboxy-terminus of 60% **E** containing domains A and C were positioned four codons upstream of the beginning of domain B, i.e., following Lys291. The original and altered nucleotide sequences of the mutagenized region and the corresponding amino acid translation are shown. . .

DETD To perform the mutagenesis, a 580 bp BamHI fragment spanning domain B from the pBR322-80% **E** clone p29D280E was subcloned into pGEM3Zf (Promega) to yield p29GEB2. (See FIG. 5.) This BamHI fragment encodes the 3' end. . .

DETD The cloned cDNA fragments encoding B domain and 80% **E** were inserted into expression vectors so as to maintain the translational frame of fusions to secretion leaders as described below.. . .

DETD The expression vector constructed to secrete classical domain B from Saccharomyces cerevisiae to include **envelope** protein amino acids 296-395 was constructed so that processing by the proteases normally involved in preproMFα_L processing would yield a. . .

DETD . . . cloning sites, and use the TRP1 gene as a selectable marker. They contain sequences derived from pBR322 to provide an **E**. coli origin of replication, the ampicillin resistance gene, and sequences derived from the 2-micron plasmid of S. cerevisiae to enable. . .

DETD . . . or Glu-Ala-DomB. For this demonstration, proteins secreted by a pLS5-DomB transformant were separated by SDS-PAGE and electroblotted to Immobilon P **membrane** (Millipore), and the amino terminal amino acid sequence was determined by microsequencing. That sequence is: H_2 N-Glu Ala Gly Met. . .

DETD . . . sodium phosphate (pH 6.7), the cells were pelleted by centrifugation and the medium was clarified by filtration through a 0.45 μm pore filter. The filtered medium was concentrated about 30-fold either by tangential flow or by centrifugal ultrafiltration using Minitan (Millipore). . .

DETD . . . color and a mostly colorless flow-through containing domain B. For DEAE chromatography, the brown-colored pooled fractions were dialyzed against 0.1 **M** acetic acid, pH 5.1 and loaded onto a 1.4×15 cm DEAE (Biorad) column. Domain B was eluted using a 0.01 **M** acetic acid, pH 5.1, 0.1 **M** NaCl step gradient.

DETD . . . domain B by overnight incubation at 4° C. and then blocked with BSA in 50 mM tris-HCl, pH 7.0, 0.15 **M** NaCl, 0.05% Tween for 1 hour at room temperature. The plates were then treated with either

DEN-2 MAb of monoclonal. . .

DETD . . . of the secreted yeast proteins and the second as 70-90% pure domain B. Treatment with 1M NaCl, 1 and 2 **M** urea, and 1% DDAPS, a zwitterionic detergent, were ineffective in completely disaggregating domain B during size exclusion chromatography. Since the. . .

DETD . . . 24 hours, the culture is supplemented with 0.01 vol of sterile 40% w/v glucose and 0.02 vol of sterile 1 **M** phosphate buffer, pH 6.7.

DETD . . . final concentrations of 1 nM each to the cleared medium, and the resulting solution is filter sterilized using a 045 **μm** pore filter **membrane** (Millipack-20 or Opticap-50, Millipore). Glycerol is added to 10% v/v to the filtrate which is then concentrated 20-30 fold using tangential flow ultrafiltration (Millipore Minitan System) with two **membrane** cartridges (four regenerated cellulose membranes) of a 10 kD MW cutoff. The retentate is kept on ice during ultrafiltration in. . .

DETD Concentrated medium of the tertiary culture is dialyzed at 4° C. us9ing a **membrane** with a 6-8 kD cutoff (Spectra/Pore-1 **Membrane**) against 10 mM acetate, pH 4.5 (4×4 liters; 2-3 hours each; 1 overnight).

DETD . . . replacing the glycerol) and cultured 48 hrs. Culture media with cells removed by sequential centrifugation and filtration through a 0.45 **μm** pore size **membrane** were buffer exchanged by diafiltration into TEEN (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EGTA, and 150. . .

DETD . . . a fusion with the $MF\alpha_L$. This construct includes those sequences that lie between domain B and the transmembrane anchor of **E**. This region contains a potential T cell epitope (Mandl et al. J Virol (1989) 63:564-571) and additional hydrophobic sequences, a. . .

DETD The domain B+stem cDNA fragment was constructed in **E**. coli cloning vectors by combining the domain B cDNA fragment and the 3' end of a 90% **E** clone. As introduced in Example 1, an **E** gene subclone representing the amino terminal 90% of **E** was constructed from DEN-2 PR159/S1 cDNA plasmid pC8 of Hahn et al. (1988, supra) using the PCR. The 90% **E** polypeptide contains all of **E** except for the C-terminal **membrane** anchor comprising two transmembrane domains. The 90% **E** cDNA clone was made as follows. The 90% **E** fragment was amplified by the PCR using pC8 as template and primers D2E937p and D2E2271m. The sequence of D2E937p is. . .

DETD . . . of #, and the two primers positioned useful restriction enzyme sites at both ends of the fragment. The PCR-amplified 90% **E** cDNA fragment was made blunt at both ends and cloned into the SmaI site of a modified pUC13 cloning vector,. . .

DETD Combining domain B and the 90% **E** 3' end made use of a unique AflIII restriction enzyme site found in most pUC-like cloning vectors and a unique AflIII site in domain B sequences. This combining was accomplished by first subcloning the 90% **E** fragment from pVZ90E into pBluescript to reverse the orientation of 90% **E** relative to the vector sequences, yielding pBS90E. Then, p29GEB24PS, containing domain B sequences in PGEM (Example 1), and pBS90E were. . . AflIII, and the vector-domain-B5' fragment and the domain-B3'-stem-vector fragment from the two digestions, respectively, were purified, ligated, and recovered in **E**. coli yielding pBS-Bstem.

DETD . . . yielded high-titre antibodies that were highly immunoreactive when used to probe Western blots displaying the same antigen or DEN-2 viral **envelope** protein.

DETD The serological diagnosis for **dengue** infection is based on the detection of anti-**dengue** IgM and IgG in primary and secondary viral infection using standard Enzyme Linked Immunosorbent Assay (ELISA) techniques. Current assays are based on the ability of anti-**dengue** immunoglobulins to recognize semi-purified virus. Primary and secondary infections can be distinguished by the IgM:IgG ratios. (Innis et al., 1989;. . .

DETD . . . in both IgM and IgG tests. In these ELISAs, the antigen was coated on plates, followed by sera positive for **dengue** antibodies and then detection by goat antihuman antibody.

DETD . . . with 100 μl of a 5 μg/ml solution of domain B. After

blocking with 5% normal goat serum, primary infected **dengue** 2 positive serum at a 1:100 dilution was added per well. The bound IgM was detected by the addition of. . .

DETD For IgG, high titer secondary **dengue** 2 infected sera were supplied at a 1:270 dilution and detected with goat anti-human IgG (Fc) conjugated to horseradish peroxidase.. . .

DETD . . . purified by the improved procedure of Example 3 is not recognized by murine polyclonal hyperimmune ascitic fluid (HMAF) to other **dengue** serotypes and to other **flaviviruses**, when assayed using a similar ELISA format. **Flavivirus** infected murine sera tested include, Japanese **Encephalitis virus**, Tick-Borne **Encephalitis virus**, **Yellow Fever virus**, Saint Louis **Encephalitis virus**, West Nile virus, three viral isolates of **dengue** serotype 1, two viral isolates of **dengue** serotype 3, and two viral isolates of **dengue** serotype 4.

DETD Sandwich assay for the detection of any domain B-containing **envelope** antigen.

DETD In an alternative enzyme immunoassay format, anti-domain B or anti-**Dengue** capture antibody, polyclonal or monoclonal, may be absorbed to the solid support, and sample containing an unknown quantity or serotype of **dengue** antigen may be added and then detected by reacting with a second anti-domain B or anti-**Dengue** antibody, either conjugated to a signal-generating enzyme or to be detected using a appropriate signal generating system, of which there are a multitude. This immunoassay is useful for the quantitation of recombinantly produced **envelope** protein or whole virus by comparing the immunoreactivity of a known concentration of domain B with that of the unknown. . . epitope binding site of the anti-domain B capture antibody can be varied to garner additional information regarding the conformation of **dengue** protein in the preparation.

DETD To perform the sandwich enzyme immunoassay, 100 μl of anti-**Dengue** monoclonal antibody 9D12 or 3H5 (Henchal, **E**. A. et al., Am J Trop Med Hyg (1985) 34:162-169) was used to coat microtiter wells. The monoclonal antibodies were. . . by Protein-A affinity chromatography and used at 10 μg/ml concentration (diluted in PBS: 50 mM sodium Phosphate, pH 7.0, 0.15 **M** NaCl). After a one hour incubation, the wells were washed three times with TBS-T (50 mM Tris-HCl PH 7.0, 0.15 **M** NaCl, 0.05% Tween-20), and blocked with 200 μl/well of 1% BSA in PBS for 1 hour at room temperature. Following. . . 200 μl/well of 1 mg/ml p-nitrophenylphosphate (pNPP) substrate in alkaline phosphatase substrate buffer (25 mM Trizma base, pH 9.5, 0.15 **M** NaCl, 5 mM $MgCl_2$; 0.02% NaN_2) was added. The plates were incubated for one hour at room temperature and. . .

DETD . . . an intracerebral injection of DEN-2 New Guinea C (NGC) strain. DomB administered in all adjuvants conferred comparable moderate survival against **dengue** virus challenges although survival was statistically significant (P<0.5 G test) only for mice immunized with DomB and Hunter's TiterMax. The. . .

DETD **E**. DomB Immunizations for Hybridoma Generation: Six BALB/c mice were immunized with 87 μg of unconjugated DomB or 174 μg KLH-conjugated. . .

DETD Cells cotransfected at ratios of 1:1, 5:1 and 20:1 were induced with 200 **μM** copper sulfate and the media and cells were harvested at days 1, 4 and 7. Western blots showed secretion of. . .

DETD Production of 60% **E** and 80% **E** in Drosophila

DETD . . . Drosophila metalothionein gene, the human tissue plasminogen activator signal and the SV-40 early polyadenylation signal, the nucleotide sequences encoding 80% **E**, **prM** 80% **E**, 60% **E** and **prM** 60% **E** are inserted and the resulting vectors used to transform Schneider cells as described in Example 9. The mature truncated forms of the **envelope** protein are secreted into the medium, or properly processed, and are conformationally correct with respect to the corresponding native portions of the **envelope** protein.

DETD . . . employed in the protocol set forth in paragraphs A and B of Example 8 produce antibodies which are neutralizing against **dengue**

virus.

DETD Expression of 80% **E** in Saccharomyces cerevisiae

DETD An expression vector (pLS6-80% **E**) was constructed for secretion of the N-terminal 80% (codons 1-395) of the DEN-2 PR-159 S1 **envelope** glycoprotein (80% **E**) from S. cerevisiae. The 80% **E** DNA sequences were obtained from plasmid p29D280E, described in example 1, by restriction endonuclease digestion with both BglII and SalI.. . . the BglII and SalI sites of pLS6, a yeast expression vector described in example 2. The resulting recombinant plasmid, pLS6-80% **E**, contains truncated **E** as a translationally in-frame fusion to the leader region of mating-factor α (MFα), a secreted yeast protein. The MFα leader. . . 66 amino acid propeptide. Cleavage of the signal peptide is expected to occur concomitantly with translocation across the endoplasmic reticulum **membrane**. Maturation of MFα normally involves removal of the propeptide by Kex2p, a golgi protease, and subsequent trimming of N-terminal (Glu/Asp)Ala dipeptides by dipeptidyl aminopeptidase (DPAP). The herein described $MF\alpha_L$ -80% **E** fusion was made such that processing of the $MF\alpha_L$ propeptide and trimming of a GluAla dipeptide results in 80% **E** with eight additional N-terminal amino acids derived from sequences present in the multiple cloning site of the pLS6 vector or in the PCR primer-adaptor used to synthesize the 80% **E** cDNA (see below).

DETD . . . ACC ATG...GGA TAA

TAG

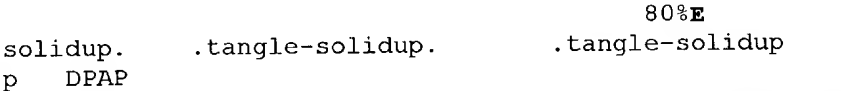
- Met-18aa Ala-65aa Glu Ala Phe Arg Ser Arg Val Pro Gly Thr Met_1..

.Gly_{395} End End

-

-

-



DETD After confirming the DNA sequence of the ligated junctions of expression vector pLS6-80% **E**, the recombinant DNA was transformed into S. cerevisiae strain GL43 (MATa trp1Δ1 ura3-52 pep4::URA3; SmithKline Beecham) according to standard protocols. . .

DETD In order to test for expression and secretion of 80% **E**, several transformants were grown as small-scale cultures (5 ml medium in 17×150 mm tubes). Single colonies were used to inoculate. . . were used to inoculate 5 ml of minimal SD medium supplemented with Casamino acids (2 g/l; Difco) and $CuSO_4$ (200 μM). This expression culture was fed with glucose (4 g/l, final concentration) and sodium phosphate (pH 6.7, 20 mM, final concentration). . . of cellular protein was prepared by lysing the yeast cells with vigorous agitation in the presence of glass beads (425-600 μm) and TEEN+PIC using a Mini Beadbeater apparatus (BioSpec Products, Bartlesville, Okla.). Samples were endoglycosidase H_f digested according to the manufacturer's. . . polyclonal serum raised against recombinant domain B (described in example 15). Negative control yeast carrying the expression vector without a **Dengue** gene insert secreted no proteins recognized by the anti-domain B serum, while the major immunoreactive band from pLS6-80% **E** medium had a relative mobility matching that of other recombinant 80% **E** proteins (see Example 17). The pLS6-80% **E** medium also contained a minor immunoreactive species with an apparent molecular weight 6-8 kD higher than 80% **E**; this is likely to be unprocessed MFα propeptide-80% **E**. The pLS6-80% **E** cellular protein extract contained many immunoreactive polypeptides not observed in negative control cells, two of which match the secreted products discussed above. A Coomassie-stained protein corresponding to recombinant 80% **E** could not be identified in either the pLS6-80% **E** secreted sample or the total cellular protein sample. This indicates the relatively low levels of recombinant protein expression in these. . .

DETD Construction of Expression Vector DPIC9-80% **E** and Secretion of 80% **E** by P. pastoris Expressing $MF\alpha_L$ -80% **E**

DETD The expression vector constructed to secrete 80% **E** from P. pastoris was engineered to express amino acids 1-395 of the DEN-2 PR-159 S1

envelope glycoprotein as a fusion to the $MF\alpha_L$. The DNA sequences encoding 80% **E** were obtained from the clone p29D280E, described in example 1, by digestion with the restriction enzymes SmaI and SalI. The. . . isolated fragment was treated with the Klenow DNA polymerase I fragment enzyme to make the SalI end blunt. This 80% **E** fragment was then cloned into the Pichia expression vector pPIC9 (Invitrogen, San Diego, Calif.) which contains the MFα secretion leader. . . ($MF\alpha_L$) sequence, SnaBI, EcoRI, and NotI cloning sites, and uses the HIS4 gene as a selectable marker. The described 80% **E** fragment was ligated with pPIC9 plasmid DNA that was previously digested with the restriction enzyme SnaBI. The orientation and genetic integrity of the resulting gene fusion expression vector, pPIC9-80% **E**, was confirmed by restriction digestion and DNA sequence analysis. The organization, partial nucleotide and predicted amino acid sequences of the $MF\alpha_L$ -80% **E** fusion gene are shown below:

DETD . . . GCC TTTAGATCTCGAGTACCCGGGACC ATG...GGA TAA
(SEQ ID NO:21 and SEQ ID NO:22)
Met-18aa-65aa
Glu Ala
PheArgSerArgValPr
oGlyThr Met_1
...Gly_{395}
END
80%**E**
.tangle-solidup. .tangle-solidup. .tangle-solidup.
- Signalase
Kex2p DPAP

DETD . . . and Kex2 cleavage sites which remove the pre and pro portions of the MFα leader peptide, respectively, are indicated. The **dengue** sequences are indicated in bold type. The Met_1 residue is the N-terminal amino acid of the **E** glycoprotein and Gly_{395} is residue 395 from the amino terminal end of the **envelope** glycoprotein. The expression of a recombinant product in Pichia from the pPIC9 vector is driven by the methanol inducible promoter. . .

DETD The pPIC9-80% **E** expression vector was transformed into spheroplasts of P. pastoris strain GS115 (his4) and transformants were selected for their ability to. . . for transformation were obtained from Invitrogen (San Diego, Calif.). Transformants were tested for their ability to express and secrete 80% **E** by growing selected clones in small cultures (5 to 50 ml). The transformants were first grown to saturation (24 to. . . medium, a unique staining band of approximately 50 kD is present in the EndoH treated lanes of all the pPIC9-80% **E** transformants. Immunoprobing with anti-domain B serum (see example 6) detected a smear ranging from 50 to 90 kD in the. . . of the corresponding immunoreactive band were detected in EndoH treated samples of cellular protein samples. The approximately 50 kD 80% **E** product produced by the $MF\alpha_L$ -80% **E** construct is consistent with the approximate molecular weight as determined by SDS-PAGE of other recombinant 80% **E** proteins (see Example 17). The amount of secreted 80% **E** in the culture medium is about 1% of the total secreted protein as estimated by the intensity of the Coomassie staining band detected. In one liter cultures, the amount of 80% **E** secreted into the culture medium was determined to be 500 ng/ml by use of a sandwich ELISA method.

DETD Construction of Expression Vector pMttbns-80% **E** and Secretion of 80% **E** by Drosophila melanogaster Schneider Cells Expressing tPA_L -80% **E**

DETD The expression vector constructed to secrete 80% **E** from Drosophila melanogaster tissue culture cells included the sequences encoding the DEN-2 PR159/S1 **envelope** glycoprotein amino acids 1-395. The DNA sequences for 80% **E** were obtained from the clone p29D280E (described in Example 1) by digestion with the restriction enzymes BglII and SalI. The released 80% **E** fragment was cloned into the BglII plus XhoI-digested D. melanogaster expression vector pMttΔXho. The expression vector pMttΔXho is a derivative. . . make

pMttΔXho, in which the BglII and another XhoI restriction endonuclease sites are unique. This construction resulted in the 80% **E** fragment being fused to the tPA_L sequence. During normal maturation of tissue plasminogen activator the 20 amino acid prepeptide region. . . amino acid propeptide region is enzymatically removed in the Golgi. The genetic integrity of the gene fusion expression vector, pMtt80% **E**, was confirmed by restriction digestion and DNA sequence analysis. The nucleotide and predicted amino acid sequences of the tPA_L -80 **E** fusion gene are shown below:

DETD . . . 20 aa - 11 aa -Gly Ala Arg Ser Arg Val Pro Gly Thr Met_1 ... Gly_{395} END

pre.tangle-solidup. pro-tPA.tangle-solidup. 80%**E**

DETD The tPA pre- and propeptide regions are delineated by pre.tangle-soliddn. and pro-tPA.tangle-soliddn., respectively, and the **dengue** sequences are indicated in bold type. The Met_1 residue is the N-terminal amino acid of the **envelope** glycoprotein and Gly_{395} is residue 395 from the amino terminal end of the **envelope** glycoprotein.

DETD The selection plasmid, pCOHygro (SmithKline Beecham), carries the **E**. coli hygromycin B phosphotransferase gene under the transcriptional control of a D. melanogaster copia transposable element long terminal repeat and. . . multiple copies of the co-transfected gene of interest. Drosophila melanogaster Schneider cells (ATCC, Rockville, Md.) were cotransfected with the pMtt80% **E** and pCOHygro plasmids at a ratio of 20:1 using the calcium phosphate coprecipitation method (Gibco BRL). Transformants were selected by. . . were split to a cell density of 2×10^6 cells/ml in serum free Excell medium (JRH Biosciences) and induced with 200 **μM** $CUSO_4$. The medium and cells from induced cells were separately harvested after 6-7 days of induction. Proteins secreted into the. . . Coomassie staining and immunoprobing of Western blots. The Coomassie blue-stained SDS-PAGE gels shows that the approximately 50 kD secreted 80% **E** product is one of the predominant proteins in the unconcentrated medium, comprising as much as 20% of the total protein.. . . revealed a single immunoreactive polypeptide of approximately 50 kD present in unconcentrated medium. In addition, immunoblots revealed that the 80% **E** produced by the tPA_L -80% **E** construct was slightly larger than that obtained upon expression of a tPA_L -prM80% **E** construct (described in detail in Example 17). This additional mass may owe to the nine adaptor amino acids at the amino terminus of 80% **E** (GARSRVPGT-(SEQ ID NO:25)80% **E**) when expressed from pMtt80% **E** versus 80% **E** expressed from pMttprM80% **E** (Example 17). The tPA propeptide, if not proteolytically removed, may also contribute to the additional molecular weight of 80% **E** expressed from pMtt80% **E**.

DETD Subcloning of **Dengue** prM100% **E** and prM80% **E** cDNAs and Mutagenesis of **E** Secretion Signal-encoding Sequence (mutSS)

DETD A cDNA clone of DEN-2 PR159/S1 designed to encode the **preMembrane**, **Membrane**, and **Envelope** genes (prM100% **E**) was constructed by PCR amplification essentially as described in Example 1 for the subcloning of 80% **E**. This cDNA clone includes nucleotides 439 to 2421 of the DEN-2 genome. The **dengue** cDNA fragment was generated using synthetic oligonucleotide primers D2pM439p and D2E2421m (see example 1 for nomenclature) and plasmid pC8 (Hahn. . . sequences described in Example 1, except that a methionine codon (ATG) was included immediately preceding the first codon of the **preMembrane** sequence (phenylalanine). The primers are:

DETD The PCR-generated prM100% **E** cDNA fragment was digested with the restriction endonuclease XbaI and ligated into the XbaI site of pBluescript SK+ (Stratagene, San. . .

DETD To generate a cDNA subclone representing prM80% **E**, a 794 bp BamHI-SalI fragment from p29prME13 representing the **envelope** carboxy terminal-encoding fragment was removed. This fragment was replaced with a 431 basepair BamHI-SalI fragment from p29D280E (described in Example

1) that encodes a 20% carboxy end truncation of the **envelope** glycoprotein. The BamHI site is a naturally occurring site within the **envelope** cDNA, and the SalI site is an engineered site that immediately follows the stop codons encoded by the PCR primers.. . . clone, p48BSprM80E, was confirmed by restriction digestion and DNA sequence analysis to encode amino acids 1 through 395 of the **envelope** glycoprotein following **prM**.

DETD Expression of the prM80% **E** cDNA in S. cerevisiae (Example 15) demonstrated absence of proteolytic processing between the **prM** and 80% **E** proteins in this yeast. To improve processing of **E** from **prM**, oligonucleotide-directed mutagenesis was performed to alter the naturally occurring signalase cleavage site between the **prM** and **E** proteins. Based on the algorithm of Von Heijne (1986, Nucl. Acids Res. 14:4683-4690), the natural DEN-2 **E** secretion signal peptide receives a poor predictive score for its function as a secretion signal. The algorithm of von Heijne. . . signal peptides range from 3.5 to 17, with a mean of .about.10. The score for the secretion signal peptide of **E** of DEN-2 PR159/S1 is 5.2, near the lower end of the range for signal peptides. In the mutagenesis, the sequence. . . and the amino acid immediately following the signalase cleavage site were altered to change these five amino acids. The modified **signal sequence** has a score of 12.4 based on von Heijne's algorithm. The original and altered nucleotide sequences of the mutagenized region. . .

DETD . . . CGC TGC (SEQ ID NO:32 and
SEQ ID NO:33)

\- Ile Ala Gly Ala Gln Ala Gln Arg Cys

\- **Membrane** .vertline. **Envelope**

\- - -

Signalase Cleavage

DETD To perform the mutagenesis, a 1,122 bp SmaI-HindIII fragment spanning the **prM-E** signalase cleavage site from the p29prME13 cDNA clone was subcloned into pAlterl (Promega, Madison, Wis.) to yield the plasmid pAltSmaH3prME. The 1,122 bp SmaI-HindIII fragment contains all of **prM** and 611 bp of the **E** sequence. The HindIII site is a naturally occurring site within the **E** sequence that is located at nucleotide 1547 of the genomic sequence. The mutagenized clone, pAltSmaH3prME(mutSS), was verified by DNA sequence. . .

DETD Construction of Expression Vectors pLS6-prM80% **E** and pLS6-**prM**(mutSS)80% **E**, Expression of $MF\alpha_L$ -prM80% **E** and $MF\alpha_L$ -**prM**(mutSS)80% **E** in Saccharomyces cerevisiae, and Secretion of 80% **E** by Saccharomyces cerevisiae Expressing $MF\alpha_L$ -**prM**(mutSS) 80% **E**

DETD For expression of DEN-2 PR159/S1 **preMembrane** protein amino acids 1-166 and **Envelope** glycoprotein amino acids 1-395 as a single continuous open reading frame in S. cerevisiae, DNA sequences encoding these proteins were. . . Example 2) that had been digested with BglII and SalI. The structure of the resulting gene fusion expression vector, pLS6-prM80% **E**, was confirmed by restriction digestion and DNA sequence analysis. The nucleotide and predicted amino acid sequences of the $MF\alpha_L$ -prM80% **E** fusion gene are shown below:

DETD . . . TTTAGATCTCGAGTACCCGGGACCATG TTT ...ACA ATG
...GGA TAA

\- Met-18aa-65aa Glu Ala PheArgSerArgValProGlyThrMet Phe_1...Thr.sub
.166 Met_1...Gly_3
95 End

\- .tangle-solidup. .tangle-solidup. .tangle-solidup.
prM 80%**E**
\- Signalase Kex2p
DPAP

DETD . . . the processing of the MFα leader peptide are indicated (See Example 1 of this application for a detailed explanation). The **dengue** sequences are indicated in bold. The Phe_1 and Thr_{166} residues are the N-terminal and C-terminal amino acid residues of **prM**, respectively. The Met_1 residue is the N-terminal amino acid of the

envelope glycoprotein and Gly_{395} is residue 395 from the amino terminal end of the **envelope** glycoprotein.

DETD The pLS6prM80% **E** plasmid was transformed into Saccharomyces cerevisiae strain GL43 (MATa ura3-52 trp1Δ1 pep4:URA3) and screened for 80% **E** expression as described in Example 1. Proteins secreted into the culture medium as well as total cellular proteins were treated. . . protein revealed a HMAF immunoreactive band of approximately 90 kD suggesting that the recombinant product had not been processed to **prM** and **E**. Probing of companion Western blots with polyclonal antisera that recognized the MFα leader peptide (from J. Rothblatt, Dartmouth University) confirmed. . . the product recognized by the anti-DEN2 HMAF was identical to that recognized by anti-MFα serum, demonstrating that the $MF\alpha_L$ -prM80% **E** fusion protein was not processed into its individual components ($MF\alpha_L$, **prM**, and 80% **E**).

DETD The unsuccessful processing of **E** from **prM** in the $MF\alpha_L$ -prM80% **E** fusion protein may be an obstacle to the proper folding and secretion of **E**. To assess whether the optimized **dengue signal sequence** (see Example 16) facilitated the processing of the **envelope** protein at the **prM-E** junction, the altered **E signal sequence** from pLS6prM(mutSS)100E-TGA was introduced into pLS6prM80% **E** to create plasmid pLS6prM(mutSS)prM80% **E**. This procedure replaced the native **E signal sequence** (Pro-Ser-Met-Thr_1 -Met+1 (SEQ ID NO:34)) with the optimized **E signal sequence** (Gly-Ala-Gln-Ala_{-1} -Gln+1 (SEQ ID NO:34)).

DETD . . . fragment between plasmids pAlterSmaH3prME(mutSS) (see Example 14) and pLS6prM100E. DNA sequencing of pLS6prM(mutSS)100E-TGA identified an unintended TGA stop codon within **E** downstream of the mutated secretion signal. To transfer the altered secretion signal encoding sequence to pLS6prM80% **E** and to separate the cDNA fragment containing the altered secretion signal of **E** from the TGA stop codon, a BglII-EcoNI fragment from pLS6prM(mutSS)100E-TGA, encompassing **prM** and the first 430 nucleotides of **E** and lacking the TGA stop codon, was transferred to plasmid pLS6prM80% **E** which had been similarly digested to yield the expression plasmid pLS6prM(mutSS)80% **E**. The sequence of the expression plasmid was confirmed by restriction digestion and DNA sequence analysis. The nucleotide and predicted amino acid sequences of the $MF\alpha_L$ -**prM** junction are identical to the sequences listed above.

DETD . . . unsupplemented minimal medium (see Example 11). Transformants were cultured, induced, and evaluated as described above for the non-mutated $MF\alpha_L$ -prM80% **E** transformants. Proteins secreted into the culture medium as well as total cellular proteins were treated with $EndoH_f$ prior to separation. . . a novel Coomassie staining band. Immunoprobing with anti-DEN2 HMAF and anti-DomB antiserum, however, revealed a small amount of processed immunoreactive **E** protein in the medium. The size of the immunoreactive protein (approximately 50 kD) was similar to the secreted protein from pLS6-80% **E** expression vector. Evaluation of intracellular expression of the fusion protein containing the optimized secretion signal by SDS-PAGE and Western blot. . . the transformed cells produce immunoreactive product recognized by anti-DEN2 HMAF and anti-DomB antiserum. Unlike the immunoreactive product seen in pLS6prM80% **E** transformants, the immunoreactive band found in pLS6(mutSS)prM80% **E** transformants was not recognized by $MF\alpha_L$ anti-serum suggesting that processing had occurred at the **prM-E** junction. Thus, the mutagenesis of the signalase cleavage site resulted in greatly enhanced processing of the $MF\alpha_L$ -prM80% **E** product at the **prM-E** junction.

DETD Construction of Expression Vector pPIC9-**prM**(mutss)80% **E** and Secretion of 80% **E** by P. pastoris Expressing $MF\alpha_L$ -**prM**(mutss) 80% **E**

DETD The expression vector constructed to express **preMembrane**-mutated

secretion signal 80% **envelope** (pPICpIM(mutSS)80% **E**) in P. pastoris from a single continuous open reading frame utilized the DEN-2 PR159/S1 **prM** and **E** gene sequences described above (Example 14). The plasmid, pPIC9-**prM**(mutSS)80% **E**, was constructed by transferring a **prM**(mutSS)80% **E** fragment from the S. cerevisiae expression plasmid pLS6prM(mutSS)80% **E** into pPIC9. The P. pastoris expression vector pPIC9 (Example 4) and the S. cerevisiae expression vector pLS6 (Example 2) both. . . endonuclease site, encoding amino acids leucine and glutamic acid, just upstream of the Kex2 protease site. The transfer of the **dengue** cDNA fragment made use of this XhoI site.

DETD Prior to transferring the **prM**(mutSS)80% **E** cDNA fragment, sequences encoding extraneous amino acids and an extraneous XhoI site at the $MF\alpha_L$ -**prM** fusion were first removed. This was accomplished by digesting pLS6-**prM**(mutSS)80% **E** with restriction endonuclease XhoI and XmaI into which was ligated a synthetic oligonucleotide duplex (5'-TCGAGAAGAGAGAAG-3' (SEQ ID NO:36) and 5'-CCGGCTTCTCTCTTC-3'. . . required for the cDNA fragment transfer from pLS6 to pPIC9. The nucleotide and predicted amino acid sequence at the $MF\alpha_L$ -**prM** fusion junction from pLS6-**prM**(mutSS)80% **E** and pLS6Δ-**prM**(mutSS)80% **E** are:

DETD $MF\alpha_L$ -**prM** junction of pLS6-**prM**(mutSS)80%**E**

```
-          XhoI                                XhoI    XmaI
- ATG ...CTC GAG AAA AGG GAG GCC TTTAGATCTCGAGTACCCGGGACCATG TTT..
                                                        (SEQ ID
                                                        NO:38 and
                                                        SEQ ID

. . . Met
                                                        ...Leu Glu
                                                        Lys Arg
                                                        Glu Ala
                                                        PheArgSerAr
                                                        gValProGlyT
                                                        hrMet
                                                        Phe1..
-                        .tangle-solidup.       .tangle-solidup.
                                                          -
                                                            Kex2p
                                                         DPAP
                                                          -
                                                        MFα.s
                                                        ub.L -prM
                                                        junction
                                                        of
                                                        pLS6.increm
                                                        ent.-prM(mu
                                                        tSS)80%E
                                                          -
                                                         XhoI
- ATG ...CTC GAG AAA AGG GAG GCC GGGACCATG TTT.. (SEQ ID NO:40 and SEQ
                                                        ID NO:41)
                                                          -. . .
```

DETD To construct the clone pPIC9-**prM**(mutSS)80% **E**, a XhoI-SalI fragment encoding **prM**(mutSS)80% **E** sequences was obtained from pLS6Δ-**prM**(mutSS)80% **E** and was inserted into the pPIC9 vector that had been digested with Xhol. The genetic integrity of the expression plasmid, pPIC9-**prM**(mutSS)80% **E**, was confirmed by restriction digestion.

DETD The pPIC9-**prM**(mutSS)80% **E** expression vector was transformed into spheroplasts of P. pastoris strain GS115 (his4), and His+ transformants were selected for their ability to grow on minimal medium without histidine supplementation. The transformants were screened for expression and secretion of 80% **E** as described in Example 12. No unique Coomassie staining bands were detected in the culture medium of either non-$EndoH_f$ or $EndoH_f$ treated samples (similar to that

observed for culture medium from prM80% **E** transformants (see Example 12). Western immunoblots of proteins from the culture medium probed with anti-domain B serum detected multiple bands with. . . corresponding immunoreactive protein were detected in EndoH treated samples of cellular proteins. We estimate that the amount of secreted 80% **E** in the culture medium is less than 1% of the total amount of secreted protein based on the observation that. . .

DETD Construction of pMttbns-prM80% **E** and Secretion of 80% **E** by Drosophila melanogaster Schneider cells Expressing tPA_L -prM80% **E**

DETD For expression of DEN-2 PR159/S1 **preMembrane** protein amino acids 1-166 and **Envelope** glycoprotein amino acids 1-395 as a single continuous open reading frame in Drosophila melanogaster Schneider 2 tissue culture cells, DNA sequences encoding these proteins were obtained by digestion of the p48BSprM80% **E** clone (described in Example 14) with the restriction enzymes BglII and SalI. This fragment was cloned into the unique BglII. . .

DETD . . . ID NO:42 and SEQ ID NO:43)

ATG ..... GGAGCCAGATCTCGAGTACCCGGGACCATG TTT ..ACA ATG ..GGA TAA

Met-20 aa- -11 aa-GlyAlaArgSerArgValProGlyThrMet Phe_1..Thr_{166} Met_1..Gly_{395} END

pre.tangle-solidup.pro-tPA.tangle-solidup.

prM 80%**E**

DETD The tPA pre- and propeptide regions are delineated by pre.tangle-soliddn. and pro-tPA.tangle-soliddn., respectively, and the **dengue** sequences are indicated in bold type. The Phe_1 and Thr_{166} residues are the N-terminal and C-terminal amino acid residues of **prM**, respectively. The Met_1 residue is the N-terminal amino acid of **envelope** glycoprotein and Gly_{395} is residue 395 from the amino terminal end of the **envelope** glycoprotein.

DETD As described previously in Example 13, Schneider 2 cells were cotransfected with pMttprM80% **E** DNA at ratios of 1:1, 5:1, and 20:1 relative to pCOHygro DNA. Transformants were induced with 200 μM $CuSO_4$ and expression of prM80% **E** was examined at various times after induction. Proteins secreted into the culture medium as well as cellular proteins were separated. . . Western blot analysis. This .about.50 kD immunoreactive band is roughly the same size as the secreted EndoH-treated product from pLS6-80% **E** transformed yeast cells (Example 11) and slightly smaller than the secreted 80% **E** from pMttbns80% **E**-transformed D. melanogaster Schneider cells (Example 13), suggesting the **Envelope** protein had been processed away from the **preMembrane** protein. (The size discrepancy between 80% **E** secreted by pMtt80% **E** and pMttprM80% **E** Schneider cells is discussed in Example 13.) Polyclonal antisera to the pr portion of **prM** (from Peter Wright, Monash University, Australia) did not recognize the .about.50 kD protein, confirming that the 80% **E** produced in the transfected cells was processed from **prM**. In fact, no evidence of a higher molecular weight band that might correspond to unprocessed prM80% **E** was detected in any sample, suggesting that the proteolytic processing of **prM** from **E** is extremely efficient in Schneider cells. The fate of the **prM** portion of the fusion remains unresolved as no distinct immunoreactive band was detected by probing with the anti-pr antisera.

DETD The secreted 80% **E** glycoprotein was partially purified (judged by the presence of a single major band on a sliver stained SDS-PAGE gel) and. . . eluted in 150 mM NaCl. The 150 mM NaCl eluant was separated on an SDS-PAGE gel and electro-transferred to Immobilon-P **membrane** (Millipore). The 80% **E** band was excised, and the N-terminal amino acids were determined by Edman sequencing. Two amino acid sequences were obtained. One,. . . expected sequence, Met-Arg-Cys-Ile-Gly-Ile (SEQ ID NO:46), supporting the interpretation that the .about.50 kD secreted immunoreactive glycoprotein is correctly processed 80% **E** of DEN-2.

DETD Sensitivity of the secreted 80% **E** to endoglycosidases was evaluated by molecular weight shift of the protein in SDS-PAGE and Western immunoblots following endoglycosidase treatment. Resistance of the secreted 80% **E** to Endoglycosidase H_f (Endo H_f ; New England

Biolabs, and sensitivity to N glycosidase F (PNGase F, New England Biolabs) digestion. . .

DETD . . . the unconcentrated medium, comprising as much as 20% of the total secreted protein. Estimates of the concentration of the 80% **E** product in unconcentrated medium based upon sandwich ELISA assays (described in detail in Example 7) and Coomassie blue staining range from 3-16 μg/ml depending on the preparation. Immunoblots probed with polyclonal anti-**dengue** 2 hyperimmune mouse ascites fluid (DEN-2 HMAF; from R. Putnak, WRAIR) demonstrated that the amount of secreted 80% **E** produced by the transfectants increased over time from day 1 post induction to 7 days post induction. The amount of 80% **E** detected intracellularly in the transfectants correlated with the cotransfection ratio, but the increase in intracellular 80% **E** with time was not as dramatic as for secreted 80% **E**, suggesting efficient secretion of 80% **E** and accumulation in the medium.

DETD Induction of Anti-**Dengue** 2 Antibodies in Mice by Pichia pastoris-secreted 80% **E**

DETD P. pastoris cells transformed with pPIC-80% **E** (described in Example 12) were induced with 0.5% methanol and the medium was collected after 40 hours of induction (for additional details on culture conditions see Example 12). The medium was filtered through a 0.5 μ**m** low protein binding filter (Opticap, Millipore), then buffer exchanged with phosphate buffered saline (10 mM sodium phosphate, 2 mM potassium phosphate, 0.15 **M** sodium chloride, and 27 mM potassium chloride, pH 7.5) and concentrated approximately 40 fold using a combination of tangential flow. . . Webster outbred mice (Simonsen) were immunized by intraperitoneal (I.P.) injection with 100 μg total protein of the crude concentrated 80% **E** medium with or without complete Freund's adjuvant. Controls for this experiment included a negative control medium prepared from a non-recombinant P. pastoris culture as described above for the 80% **E** medium. Protein precipitation was observed during the concentration of the negative control medium, consequently the final protein concentration of the concentrated medium was lower than that from the 80% **E** medium. (For this reason, 12.5 μg of total protein in Freund's complete adjuvant was used for immunization with the negative control medium.) Additional controls included saline and KLH-domain B, a recombinant **dengue** product previously shown to induce neutralizing antibodies (Example 8), both were administered with Freund's complete adjuvant. The mice received three. . .

DETD . . . given viral inoculum. Results from the ELISA and PRNT assays are summarized in Table 4. The P. pastoris expressed 80% **E** induces a potent anti-DEN2 response in mice, with ELISA titers of up to 1:102,400. The titers obtained in the presence. . .

DETD TABLE 4

Induction of Anti-DEN2 Immune Response in Mice Immunized with P. pastoris-expressed 80% **E**

mouse	antigen	adjuvant	3° titer ELISA	4° titer ELISA	4° titer $PRNT_{80}$
30-1	Saline	Freund's	<1:50	<1:100	
30-2			<1:50	<1:100	
30-3.	.	.	<1:100	<1:100	
34-5	medium		<1:100	1:100	
36-1	100 μg	Freund's	>1:6400	1:25,600	<1:10
36-2	Pichia		1:6400	1:25,600	<1:10
36-3	80% **E**		>1:6400	1:25,600	<1:10
36-4	total		1:100	1:100	<1:10
36-5	medium		1:6400	1:102,400	<1:10
37-1	100 μg	None	<1:100	1:100	
37-2	Pichia		1:1600	1:6400	
37-3	80% **E**		1:100	1:400	
37-4	total		1:400	1:6400	

37 3 medium 1:100 <1:100

DETD Induction of **Dengue** Virus-neutralizing Antibodies by Immunizing Mice with 80% **E** Secreted by Drosophila melanogaster Schneider Cells Expressing tPA_L -prM80% **E** and tPA_L -80% **E**

DETD Schneider cells, transformed with pMtt-prM80% **E** and pMtt-80% **E** expressing the tissue plasminogen activator leader fusion proteins tPA_L -prM80% **E** and tPA_L -80% **E**, respectively (Described in detail in Examples 17 and 13, respectively), were cultured in serum-free medium (Excell; JRH Biosciences) and induced. . . by centrifugation at 1000 X G in a Beckman TJ-6 refrigerated centrifuge and the media were filtered through a 0.2 μm cellulose acetate filter (Nalgene). The media containing the recombinant 80% **E** were concentrated 20-fold using centrifugal concentrators (Centriprep 30; Amicon) and assayed by ELISA (described in detail in Example 7) and. . .

DETD . . . summarized in Tables 5 and 6. In both series of immunizations, the mice immunized with the crude media containing 80% **E**, expressed cotranslationally with **prM** or independently without **prM**, developed high titer, virus-neutralizing antibodies. These titers are higher than any previously reported titers for any immunogen produced from any **flavivirus**, suggesting the utility of these immunogens as efficacious vaccine candidates.

DETD TABLE 5

Immune Response of Mice Immunized with Crude Drosophila Media Containing **Dengue** 2 Virus 80% **E** Expressed as a **prM** 80% **E** Fusion

mouse	antigen	adjuvant	2° titer[a] ELISA	3° titer[b] ELISA	$PRNT_{80}$ titer[c] ELISA titer[c]
20-1		Saline			
		Freund's			
			<1:50		
				<1:50	<1:100
. . .			<1:50	<1:100	<1:10
21-3	medium		<1:50	<1:50	<1:100 <1:10
21-4			<1:50	<1:50	<1:100 <1:10
21-5			<1:50	<1:50	<1:100 <1:10
22-1	**prM** 80% **E**	Freund's	1:3200	>1;25,600	1:102,400 1:2560
22-2	secreted		>1:800	>1:6,400	1:25,600 1:2560
22-3	medium		>1:200	>1:1,600	1:25,600 1:2560
22-4			<1:50	>1:102,400. . .	

DETD TABLE 6

Immune Response of Mice Immunized with Crude Drosophila Media Containing **Dengue** 2 Virus 80% **E** Expressed with or without **prM**

mouse	antigen	adjuvant	2° titer[a] ELISA	3° titer[b] ELISA	Final titer[c] ELISA
25-1	Saline	Freund's	<1:50	>1:50	<1:10
25-2			<1:50	>1:50. . .	>1:6400 1:409,600 1:40
27-5			>1:6400	1:409,600	<1:500
28-1	100 μg	Freund's	DEAD	NT	NT
28-2	Drosphila		>1:6400	1:102,400	1:8000
28-3	**prM** 80% **E**		>1:6400	1:409,600	1:8000
28-4	total		>1:6400	1:102,400	1:4000
28-5	medium		>1:6400	1:102,400	1:1000
29-1	100 μg	Freund's	1:6400	1:6400	1:500
29-2	Drosophila		1:6400	1:102,400	1:4000
29-3	80% **E**		<1:100	1:25,600	1:1000
29-4	total		>1:6400	1:25,600	1:8000

29 5 medium >1.0400 1.102,400 1.4000

[a] Determined following the 2nd injection.

[b]. . .

DETD Protection from **Dengue** Virus Challenge by Immunizing Mice with 80% **E** Secreted by Drosophila melanogaster Schneider Cells Expressing tPA_L -prM80% **E** or tPA_L -80% **E**

DETD Schneider cells, transformed with pMtt-prM80% **E** and pMtt-80% **E** expressing the tissue plasminogen activator leader fusion proteins tPA_L -prM80% **E** and tPA_L -80% **E** respectively (described in detail in Examples 17 and 13, respectively), were cultured in serum-free medium (Excell; JRH Biosciences) and induced. . . by centrifugation at 1000 X G in a Beckman TJ-6 refrigerated centrifuge and the media were filtered through a 0.2 μm cellulose acetate filter (Nalgene). The media were concentrated 20-fold using centrifugal ultrafiltration (Centriprep 30, Amicon) and assayed by ELISA and. . . of each preparation (corresponding to 70 μg total protein from negative control medium, 230 μg total protein from tPA_L -80% **E** medium, and 150 μg total protein from tPA_L -prM80% **E** medium) were used to subcutaneously inoculate groups of 10 mice each, using Alum as adjuvant. An identical second dose was. . .

DETD . . . the remaining two mice recovered by day 15 post-challenge. In contrast, of the 10 mice immunized with the tPA_L -80% **E**, only four exhibited any symptoms of infection, and eight of 10 survived the challenge. Similarly, nine of 10 mice immunized with tPA_L -prM80% **E** survived the challenge, although seven of these mice exhibited mild symptoms of infection during the monitoring period. These survival data are illustrated in FIG. 13, and show that both Drosophila-expressed 80% **E** antigens efficiently protected mice from viral challenge. These results emphasize the utility of the Drosophila cell expressed 80% **E** **dengue** immunogens as vaccine candidates.

DETD Construction of DEN-2 N-terminal 60% **E** and prM60% **E** cDNA Fragments

DETD A subclone encoding the N-terminal 60% of **E** was constructed using p29GEB24PS and p29D280E. Example 1 of the parent application--describes construction of p29GEB24PS. Plasmid p29GEB24PS holds a BamHI fragment insert containing, in part, DEN-2 **E** sequences (nucleotides 1696-2121) starting at a DEN-2 genomic BamHI site and ending with the Gly395 codon, followed immediately by two. . .

DETD The N-terminal 80% **E** insert in p29D280E was then converted to a 60% **E** insert by replacing a restriction fragment encoding the 3' end of 80% **E** with a restriction fragment from p29GEB24PS encoding the 3' end of 60% **E**. To accomplish this, DNA of p29GEB24PS was digested with BamHI, the .about.590 bp BamHI fragment was isolated by agarose gel. . . then digested with SalI, and finally the 119 bp BamHI-SalI fragment released from the .about.590 bp BamHI fragment and containing **dengue** nucleotides 1696-1809 was isolated by agarose gel electrophoresis and ligated into p29D280E prepared as follows. Plasmid p29D280E was digested with BamHI, which cuts the BamHI site (**dengue** nucleotides 1696-1701) within 80% **E**, and with SalI, which cuts immediately 3' of 80% **E** and also within the vector, pBR322, 422 base pairs distal to the 3' end of the 80% **E** fragment. Following ligation, the desired product, a plasmid containing the cDNA encoding the N-terminal 60% of **E** in pBR322 (p29D260E), was recovered by transformation of **E**. coli with the ligation mixture and screening transformant colonies for plasmids of the appropriate size and restriction digestion pattern. Proper. . .

DETD To construct a cDNA encoding **prM** and the amino terminal 60% of **E** (prM60% **E**), we used a strategy identical to that used to construct prM80% **E** (Example 14). The prM100% **E** plasmid, p29prME13, was digested with BamHI and SalI to release the 794 bp 3' end fragment of **E**, which was then replaced with the 119 bp BamHI-SalI fragment encoding a 40% carboxy-end truncation of **E** from p29D260E. The resulting truncated cDNA clone, p48BSprM60E, encodes a **prM**-60% **E** fusion ending with Lys291 of **E** and was confirmed by restriction digestion and DNA sequence analysis.

DETD Construction of Expression Vector pLS6-60% **E** and Secretion of 60% **E** by Saccharomyces cerevisiae Expressing MFα-60% **E**

DETD An expression vector (pLS6-60% **E**) was constructed for secretion of the N-terminal 60% (codons 1-291, 60% **E**) of the DEN-2 PR-159 S1 **envelope** glycoprotein from S. cerevisiae. The 60% **E** DNA sequences were obtained from plasmid p29D260E, described in example 21, by restriction endonuclease digestion with both BglII and SalI.. . . subcloned into the BglII and SalI sites of pLS6, a yeast expression vector described in example 2. The $MF\alpha_L$ -60% **E** fusion was made such that processing of the MFα propeptide and trimming of a Glu-Ala dipeptide results in 60% **E** with eight additional N-terminal amino acids encoded by sequences present in the multiple cloning site of the pLS6 vector and the **E** gene PCR primer adaptor (see below).

DETD . . . - Met-18aa Ala-65aa Glu Ala Phe Arg Ser Arg Val Pro Gly Thr Met_1...

Lys_{291} End End

\- .tangle-solidup. .tangle-solidup. .tangle-solidup. 60%**E**

\- Signalase Kex2p DPAP

DETD After confirming the DNA sequence of the ligated junctions of expression vector pLS6-60% **E**, the recombinant DNA was transformed into S. cerevisiae strain GL43 (MATa trp1Δ1 ura3-52 pep4::URA3; SmithKline Beecham) according to standard protocols. . .

DETD In order to test for expression and secretion of 60% **E**, several transformants were grown in small-scale cultures (5 ml medium in 17×150 mm tubes). Single colonies were used to inoculate. . . were used to inoculate 5 ml of minimal SD medium supplemented with Casamino acids (2 g/l; Difco) and $CuSO_4$ (200 μM). This expression culture was fed with glucose (4 g/l, final concentration) and sodium phosphate (pH 6.7, 20 mM, final concentration). . . of cellular protein was prepared by lysing the yeast cells with vigorous agitation in the presence of glass beads (425-600 μm) and TEEN+PIC (TEEN with 1 μg/ml each of pepstatin and leupeptin and 1 mM phenylmethylsulfonylfluoride) using a Mini Beadbeater apparatus. . .

DETD Negative control yeast carrying the expression vector without a **Dengue** gene insert secreted no proteins recognized by anti-DEN2 HMAF, while pLS6-60% **E** medium contained several immunoreactive species. The major band presumably represents full-length 60% **E** since its apparent molecular weight is approximately 10 kD less than that of recombinant 80% **E**. A protein band comigrating with this immunoreactive material was visible in Coomassie-stained gels of proteins secreted by pLS6-60% **E** transformants, but this band was absent in medium of negative control transformants. The immunoblot of proteins secreted by pLS6-60% **E** transformants evidenced a minor band of apparent molecular weight 6-8 kD larger than 60% **E**; this likely represents unprocessed MFα propeptide-60% **E**. The cellular protein extract of pLS6-60% **E** transformants contained many immunoreactive polypeptides not observed in negative control cells; two of these match the secreted products discussed above.

DETD Construction of Expression Vectors pLS6-prM60% **E** and pLS6-**prM**(mutSS)60% **E**, Expression of $MF\alpha_L$ -prM60% **E** and $MF\alpha_L$ -**prM**(mutSS)60% **E** in Saccharomyces cerevisiae, and Secretion of 60% by Saccharomyces cerevisiae Expressing $MF\alpha_L$ -**prM**(mutSS)60% **E**

DETD For expression of DEN-2 PR159/S1 **premembrane** protein amino acids 1-166 and **envelope** glycoprotein amino acids 1-291 as a single contiguous open reading frame in S. cerevisiae, DNA sequences encoding these proteins were. . . Example 2) that had been digested with BglII and SalI. The structure of the resulting gene fusion expression vector, pLS6-prM60% **E**, was confirmed by restriction digestion and DNA sequence analysis. The N-terminal $MF\alpha_L$ -**prM** fusion amino acid sequence of the $MF\alpha_L$ -prM60% **E** fusion protein are identical

to those described for MFα_L -**prM**(mutSS)60% **E** fusions (Example 15), while the C-terminal amino acid of the fusion protein is Lys_{291} of the **dengue envelope** glycoprotein.

DETD The pLS6-prM60% **E** plasmid was transformed into S. cerevisiae strain GL43 (MATa ura3-52 trp1Δ1 pep4::URA3) and screened for 60% **E** expression as described in Example 11. Proteins secreted into the culture medium as well as cellular proteins were treated with. . . analyzed by both Coomassie staining of polyacrylamide gels and immunoprobing of Western blots. Similar to the expression of secreted 80% **E** from pLS6-prM80% **E** (Example 15), secreted 60% **E** was not detected on Coomassie stained polyacrylamide gels nor Western blots. An immunoblot of intracellular protein confirmed that the construct (MFα_L -prM60% **E**) was expressed, but the fusion product had not been processed to **prM** and **E** (evaluation was performed as described in Example 15).

DETD Because the **dengue E signal sequence** itself may limit processing of the prME fusion proteins, expression of MFα_L -**prM**(mutSS)60% **E** was evaluated. The BglII-EcoNI fragment from pLS6-**prM**(mutSS)100% **E**-TGA encoding the altered secretion signal peptidase cleavage site (see Example 15) was used to replace the homologous fragment from the pLS6-prM60% **E** to produce pLS6-**prM**(mutSS)60% **E**. The sequence of the expression plasmid was confirmed by restriction digestion and DNA sequence analysis. Plasmid pLS6prM(mutSS)60% **E** was transformed into the S. cerevisiae GL43 strain and transformants were selected as described in Example 11.

DETD . . . were cultured, induced, and evaluated as described in Examples 11 and 15. In contrast to the expression of MFα_L -prM60% **E**, Western blot analysis of total intracellular proteins from pLS6-**prM**(mutSS)60% **E** transformants demonstrated that the transformed cells produce an approximately 40 kilodalton product recognized by anti-DEN2 HMAF. For analysis of secreted proteins, media from induced cultures were concentrated, treated with endoglycosidase H_f, and analyzed on Western blots for **E** antigen. A small amount of processed 60% **E** could be identified in the culture medium upon immunoprobing with anti-DEN2 HMAF. Thus, the mutagenesis of the signalase cleavage site resulted in greatly enhanced processing of the MFα_L -**prM**(mutSS)60% **E** product at the **prM-E** junction which produced secretion of a processed 60% **E** from the MFα_L -**prM**(mutSS)60% **E** in S. cerevisiae.

DETD Construction of Expression Vector pPIC9-60% **E** and Secretion of 60% **E** by P. pastoris Expressing MFα_L -60% **E**

DETD The expression vector constructed to secrete 60% **E** from P. pastoris, pPIC9-60% **E**, included the DEN-2 PR-159 S1 **envelope** glycoprotein amino acids 1-291. As a precursor to this 60% **E** expression vector, a modified pLS6-60% **E** plasmid (pLS6-alt60% **E**), encoding a fusion between MFα_L and the amino terminal 60% of the **dengue envelope**, was constructed that encodes fewer non-**dengue** amino acids between the MFα_L and **E** segments. The 60% **E** cDNA fragment from pLS6-alt60% **E** was then transferred to pPIC9.

DETD The sequences in pLS6-alt60% **E** encoding the **dengue E** protein amino terminus were derived from pLS6-2×80E, which encodes a tandemly arrayed dimer of 80% **E** that are linked by a synthetic linker peptide. To convert pLS6-2×80E to pLS6-alt60% **E**, pLS6-2×80E was digested with EcoNI, which cuts within the first member of the 80% **E** dimer, and SalI, which cuts immediately downstream of the dimer, thereby removing the 3' portion of the first member of. . . 80E dimer and the second member of the 80E dimer entirely. In its place was ligated an EcoNI-SalI fragment from pLS6-**prM**(mutSS)60E (see Example 23), encoding the 3' portion of 60% **E**, to complete construction of pLS6-alt60% **E**.

DETD The minimizing of non-**dengue** codons between MFα_L and **E**, found in pLS6-2×80E, were preserved in pLS6-alt60% **E**, and, subsequently, in pPIC9-60% **E**. The codons between MFα_L and

E were minimized, owing to use of the XmaI site in p29b60E (XmaI is the first adaptor restriction enzyme site upstream of **E**; see Example 1) and the StuI site in pLS6. To ligate the XmaI 5' end of **E** to the StuI site in the $MF\alpha_L$, the XmaI site was treated with Klenow polymerase to make the end blunt. . .

DETD To transfer 60% **E** from pLS6-alt60% **E** to pPIC9, pLS6-alt60E was digested by XhoI plus SalI, which released a fragment that includes a portion of the MFα leader and the entire 60% **E** coding region. This fragment was ligated with the Pichia expression vector pPIC9 (Invitrogen, San Diego, Calif.; described in Example 12). . . complete MFα leader sequence including the Kex2 cleavage site. The genetic integrity of the resulting gene fusion expression vector, pPIC9-60% **E**, was confirmed by restriction digestion and DNA sequence analysis. The partial nucleotide and predicted amino acid sequences of the $MF\alpha_L$ -60% **E** fusion gene are shown below:

DETD . . . and Kex2 cleavage sites which remove the pre and pro portions of the MFα leader peptide, respectively, are indicated. The **dengue** sequences are indicated in bold type. The Met_1 residue is the N-terminal amino acid of the **E** glycoprotein and Lys_{291} is residue 291 from the amino terminal end of the **envelope** glycoprotein. The expression of a recombinant product in Pichia from the pPIC9 vector is driven by the methanol inducible promoter. . .

DETD The pPIC9-60% **E** expression vector was transformed into spheroplasts of P. pastoris strain GS115 (his4) and transformants were selected for their ability to. . . for transformation were obtained from Invitrogen (San Diego, Calif.). Transformants were tested for their ability to express and secrete 60% **E** by growing selected clones in small cultures (5 ml). The transformants were grown to saturation (24 to 36 hrs.) in. . . Mass.) and separated by SDS-PAGE. Western immunoblots probed with DEN-2 HMAF indicated that the recombinants expressed significant levels of 60% **E**. Protein gels analyzed by Coomassie staining also showed strong levels of 60% **E** expression and secretion.

DETD Construction of Expression Vector pPIC9-**prM**(mutSS)60% **E** and Secretion of 60% **E** by P. pastoris Expressing $MF\alpha_L$ -**prM**(mutSS)60% **E**

DETD To construct clone pPIC9-**prM**(mutSS)60% **E**, a strategy identical to that describe in Example 16 was used. Clone pLS6-**prM**(mutSS)60% **E** (described in Example 23) was digested with restriction endonuclease XhoI and XmaI and sequences within the $MF\alpha_L$ and **dengue** cloning adaptor were replaced by oligonucleotides to remove an extraneous XhoI site, to preserve a critical XhoI site, and to regenerate the Kex2 protease process site. The XhoI-SalI **prM**(mutSS)60% **E** fragment from pLS6Δ-**prM**(mutSS)60% **E** was ligated into the unique XhoI site of pPIC9. The nucleotide and amino acid sequences at the N-terminus of the fusion protein are identical to that shown in Example 16 for pLS6Δ-**prM**(mutSS)80% **E**. The structure of the Pichia expression vector pPIC9-**prM**(mutSS)60% **E** was confirmed by restriction digestion and DNA sequence analysis.

DETD The pPIC9-**prM**(mutSS)60% **E** expression vector was transformed into spheroplasts of P. pastoris strain GS115 (his4), and transformants were selected and evaluated as described. . .

DETD Construction of pMttbns-prM100% **E**, Expression of tPa-prM100% **E** by Drosophila melanogaster Schneider Cells, and Induction of a Virus Neutralizing Response by Immunizing Mice with tPa-prM100% **E Membrane** Preparations

DETD For expression of DEN-2 PR159/S1 **preMembrane** protein amino acids 1-166 and **Envelope** glycoprotein amino acids 1-495 as a single continuous open reading frame in Drosophila melanogaster Schneider 2 tissue culture cells, DNA. . .

DETD . . . and SEQ ID NO:52)

ATG ..... GGAGCCAGATCTCGAGTACCCGGGACCATG TTT ..ACA ATG ..GCC TAA

Met-20 aa-

ValProGlyThrMet
$Phe_1..Thr_{166}$
$Met_1..Ala_{495}$
END

pre.tangle-solidup.pro-tPA.tangle-solidup.

prM

100%**E**

DETD The TPA pre- and propeptide regions are delineated by pre.tangle-soliddn. and pro-tpA.tangle-soliddn., respectively, and the **dengue** sequences are indicated in bold type. The Phe_1 and Thr_{166} residues are the N-terminal and C-terminal amino acid residues of **prM**, respectively. The Met_1 residue is the N-terminal amino acid of **envelope** glycoprotein and Ala_{495} is residue 495 from the amino terminal end of the **envelope** glycoprotein (the carboxy-terminal residue).

DETD As described previously in Example 13, Schneider 2 cells were cotransfected with pMttprM100% **E** DNA at ratios of 1:1, and 5:1, relative to pCOHygro DNA and selected for growth in medium containing 300 µg/ml hygromycin. Transfectants were induced with 200 µM $CUSO_4$ and expression of prM100% **E** was examined at various times after induction. Proteins secreted into the culture medium as well as cellular proteins were separated. . . a unique protein band either intracellularly or in the culture medium. However, a novel band was recognized intracellularly by polyclonal anti-**dengue** 2 hyperimmune mouse ascites fluid (anti-DEN-2 HMAF, gift of R. Putnak, WRAIR) in Western blot analysis. This .about.60 kD immunoreactive band comigrated with viral **Envelope** derived from **dengue**-2 infected mosquito C6/36 cells suggesting the recombinant 100% **E** protein had been processed away from the **premembrane** protein. Polyclonal antisera to the pr portion of **prM** (from Peter Wright, Monash University, Australia) recognized a .about.20 kD protein, which comigrated with viral **prM**, confirming that the 100% **E** produced in the transfected cells was processed from **prM**. In fact, no evidence of a higher molecular weight band that might correspond to unprocessed prM100% **E** was detected in any sample, suggesting that the proteolytic processing of **prM** from **E** is extremely efficient in Schneider cells. No 100% **E** was detected in the culture medium indicating that 100% **E** remains anchored in cell-associated membranes.

DETD Immunoblots probed with anti-DEN-2 HMAF demonstrated that the amount of intracellular 100% **E** produced by the transfectants increased over time from day 1 post induction to 7 days post induction. The amount of 100% **E** detected intracellularly in the transfectants correlated with the cotransfection ratio. Sensitivity of the intracellular 100% **E** to endoglycosidases was evaluated by molecular weight shift of the protein in SDS-PAGE and Western immunoblots following endoglycosidase treatment. Partial resistance of the recombinant 100% **E** to Endoglycosidase H_f (Endo H_f ; New England Biolabs) digestion indicated that the product contains N-linked glycosylation, and that the. . .

DETD Schneider cells, transformed with pMtt-prM100% **E** expressing the tissue plasminogen activator leader fusion protein tPA_L -prM100% **E** were cultured in serum-free medium (Excell; JRH Biosciences) and induced by addition of $CuSO_4$ to a final concentration in the. . . Negative control cells transformed with pCOHygro only (see Example 13) were cultured, induced, and harvested as described for the prM100% **E**-expressing cells. The immunogens were assayed by Western blot prior to immunization of mice.

DETD Outbred Swiss Webster mice (Simonsen) were immunized intraperitoneally (I.P.) with 75 µg total protein of the prM100% **E membrane** preparation in Freund's complete adjuvant. Control animals were immunized with either 75 µg of total protein from the negative control **membrane** preparation or with saline only, each in Freund's complete adjuvant. The mice received three I.P. boosts, consisting of one half. . .

DETD . . . In Example 10. Results for the ELISA and PRNT assays are summarized in Table 7. The mice immunized with the **membrane** preparation containing 100% **E** developed virus-neutralizing antibodies despite the crude nature of the immunogen, suggesting the utility of these immunogens as efficacious vaccine candidates.

DETD TABLE 7

Immune Response of Mice Immunized with Recombinant **Dengue** prM100% **E Membrane** Preparation

mouse	antigen	adjuvant	2° titer[a] ELISA	3° titer[b] ELISA	final titer ELISA titer[b]	$PRNT_{80}$
20-1	Saline	Freund's	<1:50	<1:50	<1:100	
. . .			<1:100	<1:10		
20-4			<1:50	<1:50	<1:100	<1:10
20-5			<1:50	<1:50	<1:100	<1:10
23-1	pCoHygro	Freund's	<1:50	<1:50	<1:100	<1:10
23-2	**membrane**		<1:50	<1:50	<1:100	<1:10
23-3			<1:50	<1:50	<1:100	<1:10
23-4			<1:50	<1:50	<1:100	<1:10
23-5			<1:50	<1:50	<1:100	<1:10
24-1	**prM** 100% **E**	Freund's	1:200	1:1,600	>1:25600	1:320
24-2	**membrane**		<1:50	>1:1600	>1:1600	1:320
24-3			<1:50	>1:6000	>1:1600	1:80
24-4			<1:50	>1:6000	>1:25600	1:640
24-5			<1:50	<1:50	1:400	1:20

[a]. . .

DETD . . . acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: cDNA
- - (vi) ORIGINAL SOURCE:
(A) ORGANISM: **Dengue** vi - #rus
(B) STRAIN: Serotype 2 - #(Den-2)
- - (vii) IMMEDIATE SOURCE:
(B) CLONE: Den-2 PR159/ - #S1
. . . (A) NAME/KEY: misc__ - #feature
(B) LOCATION: 343
(D) OTHER INFORMATION: - #/note= "Start of coding strand sequence - #for **preMembrane**"
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 616
(D) OTHER INFORMATION: - #/note= "Start of coding strand sequence - #for **Membrane**"
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 841
(D) OTHER INFORMATION: - #/note= "Start of coding strand sequence - #for **Envelope**"
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 2326
(D) OTHER INFORMATION: - #/note= "Start of coding. . . acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: cDNA
- - (vi) ORIGINAL SOURCE:

```
          (A) ORGANISM: Dengue v1    #1us
          (B) STRAIN: Serotype 2( - #DEN-2)
  - -     (vii) IMMEDIATE SOURCE:
          (B) CLONE: Den-2 PR159/ - #S1
.  .  .  (A) NAME/KEY: misc__ - #feature
          (B) LOCATION: 343
          (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #for preMembrane"
  - -     (ix) FEATURE:
          (A) NAME/KEY: misc__ - #feature
          (B) LOCATION: 616
          (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #of Membrane"
  - -     (ix) FEATURE:
          (A) NAME/KEY: misc__ - #feature
          (B) LOCATION: 841
          (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #of Envelope"
  - -     (ix) FEATURE:
          (A) NAME/KEY: misc__ - #feature
          (B) LOCATION: 2326
          (D) OTHER INFORMATION: - #/note= "Start of coding.  .  .  - #C CCA
      CCA ACA GCA GGG
    192
 Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Il - #e Pro Pro Thr Ala Gly
     50              - #     55              - #     60
  - - ATA TTA AAA AGA TGG GGA.  .  .  - #T AAG ACA AAG GAC GGC
    432
 Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Ph - #e Lys Thr Lys Asp Gly
    130              - #   135              - #   140
  - - ACG AAC ATG TGT ACC CTC.  .  .
DETD    .  .  .  - #A ATG GAT CTG GAA AAA
   1872
 Asp Gly Ser Pro Cys Lys Ile Pro Phe Glu Il - #e Met Asp Leu Glu Lys
    610              - #   615              - #   620
  - - AGA CAT GTT TTG GGC CGC.  .  .  - #C GTC ACA GAT AAC GTG
   2400
 Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Ph - #e Val Thr Asp Asn Val
 785                7 - #90                7 - #95                8 -
#00
   - - CAT ACA TGG ACA.  .  .  - #A GAG AAA GCT TCT TTC
   2976
 Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Il - #e Glu Lys Ala Ser Phe
            980      - #          985      - #          990
  - - ATT GAA GTC AAA AGT TGC.  .  .  - #A ACG GAA TGG TGT TGT
   3264
 Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Il - #e Thr Glu Trp Cys Cys
        1075         - #       1080          - #      1085
  - - CGA TCT TGC ACA CTA CCA.  .  .  - #         40         - #         45
  - - Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Il - #e Pro Pro Thr Ala Gly
     50              - #     55              - #     60
  - - Ile Leu Lys Arg Trp Gly.  .  .  - #       120          - #       125
  - - Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Ph - #e Lys Thr Lys Asp Gly
    130              - #   135              - #   140
  - - Thr Asn Met Cys Thr Leu.  .  .  - #       600          - #       605
  - - Asp Gly Ser Pro Cys Lys Ile Pro Phe Glu Il - #e Met Asp Leu Glu Lys
    610              - #   615              - #   620
  - - Arg His Val Leu Gly Arg.  .  .  - #   775              - #   780
  - - Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Ph - #e Val Thr Asp Asn Val
 785                7 - #90                7 - #95                8 -
#00
   - - His Thr Trp Thr.  .  .  - #               970  - #               975
  - - Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Il - #e Glu Lys Ala Ser Phe
           980      - #          985      - #          990
  - - Ile Glu Val Lys Ser Cys.  .  .  - #          1065      - #          1070
```

Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Il #e Thr Glu Trp Cys Cys
1075 - # 1080 - # 1085
- - Arg Ser Cys Thr Leu Pro. . .
DETD . . . (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: peptide
- - (ix) FEATURE:
(A) NAME/KEY: Cleavage-sit - #e
#20) (B) LOCATION: (19
(D) OTHER INFORMATION: - #/note= "Signalase cleavage"
- - (ix) FEATURE:
(A) NAME/KEY: Cleavage-sit - #e
#86) (B) LOCATION: (85
(D) OTHER INFORMATION: - #/note= "Kex2p cleavage"
- - (ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..19
. . . #50 1 - #55 1 -
#60
- - Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile Il - #e Ile Gly Val Glu
Pro
165 - # 170 - # 175
- - Gly Gln Leu Lys Leu Asp Trp. . . base - #pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (vi) ORIGINAL SOURCE:
(A) ORGANISM: **Dengue** vi - #rus
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 27..46
(D) OTHER INFORMATION: - #/note=. . .
DETD . . . GGG ACC ATG TT - #T -
33
Met Leu Glu Lys Arg Glu Ala Gly Thr Met Ph - #e
20 - # 25
- - - - (2) INFORMATION FOR SEQ ID NO:41:
- - (i) SEQUENCE CHARACTERISTICS:
(A). . . (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:
- - Met Leu Glu Lys Arg Glu Ala Gly Thr Met Ph - #e
1 5 - # 10
- - - - (2) INFORMATION FOR SEQ ID NO:42:
- - (i) SEQUENCE CHARACTERISTICS:
. . .

1. An immunogenic composition which generates protective, neutralizing antibody responses to a **Flavivirus** in a murine host which responses confer protection against intracerebral challenge by the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of Japanese **encephalitis virus** (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne **encephalitis virus** (TBE) which composition contains an adjuvant; and a portion of the **envelope** protein (**E**) of the **Flavivirus** strain against which said responses are sought, which portion is 80% **E**, wherein said 80% **E** represents that portion of the **envelope** protein that constitutes 80% of its length starting from amino acid 1 at its N-terminus and which portion has been. . .

4. An immunogenic composition which generates a neutralizing antibody response to a **Flavivirus** in a murine host against the homologous **Flavivirus**, said strain of **Flavivirus** selected from the group consisting of a strain of **dengue**, a strain of Japanese **encephalitis virus** (JEV), a strain of **yellow fever virus** (YF), and a strain of tick-borne **encephalitis virus** (TBE) which composition contains an adjuvant; and a portion of the **envelope** protein (**E**) of the **Flavivirus** strain against which generation of said response is sought, which portion is 80% **E**, wherein said 80% **E** represents that portion of the **envelope** protein that constitutes 80% of its length starting

from amino acid 1 at its N terminus, and which portion has been. . .
6. The immunogenic composition of claim 4 wherein said **Flavivirus** is a **dengue** virus.

7. The immunogenic composition of claim 4 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA_L).

9. A method to generate a neutralizing antibody response in a non-human subject against a **Flavivirus** strain, said strain selected from the group consisting of a strain of **dengue**, a strain of YF, a strain of JEV, and a strain of TBE, which method comprises administering to a non-human. . .
10. The method of claim 9 wherein said **Flavivirus** is a **dengue** virus.

11. The immunogenic composition of claim 1 wherein the 80% **E** is encoded in a DNA construct operably linked downstream from a human tissue plasminogen activator prepropeptide secretion leader (tPA_L) sequence.
. . .
12. The immunogenic composition of claim 1 wherein said **Flavivirus** is a **dengue** virus.

13. The immunogenic composition of claim 2 wherein the Flaviririus is a **dengue** virus.

L14 ANSWER 6 OF 15 USPATFULL on STN
2000:142128 Methods of preparing carboxy-terminally truncated recombinant **flavivirus envelope** glycoproteins employing drosophila melanogaster expression systems.
Ivy, John, Kailua, HI, United States
Nakano, Eilen, Honolulu, HI, United States
Clements, David, Honolulu, HI, United States
Hawaii Biotechnology Group, Inc., Aiea, HI, United States (U.S. corporation)
US 6136561 20001024
APPLICATION: US 1997-937195 19970925 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, Japanese encephalitis (JE) virus, tick-borne **encephalitis virus** (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), **membrane** (**M**), and **envelope** (**E**) proteins. The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the **E** glycoprotein that retain their native configuration and attendant properties associated therewith (i.**e**., ability to induce neutralizing antibody responses). To date, recombinantly produced **E** glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the **E** glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of **E** containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in Drosophila melanogaster Schneider cell lines. The recombinant proteins produced by this expression system should prove useful, inter alia, as immunogens and diagnostic reagents.
CLM What is claimed is:

1. An expression system for the recombinant production and secretion of a portion of an **envelope** (**E**) protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese **encephalitis virus** (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which expression system comprises Drosophila cells modified to contain a DNA molecule which comprises (a) a first nucleotide sequence encoding said portion of said **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395, and (b) a second nucleotide sequence which encodes a secretory leader sequence or a secretory **signal sequence** operably linked to said first nucleotide sequence and positioned so as to produce a fusion protein when said first and said second nucleotide sequences are expressed in a eucaryotic cell, said encoding sequences operably linked to control sequences capable of effecting expression of said encoding nucleotide sequences in eucaryotic cells.

2. The expression system of claim 1 wherein said secretory leader sequence is human tissue plasminogen activator prepropeptide secretion leader (tPA_L) and optionally includes the **premembrane** leader of the **E** protein.

3. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese **encephalitis virus** (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 1 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

4. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese **encephalitis virus** (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 2 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

5. The expression system of claim 1 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

6. The method of claim 3 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

7. The method of claim 4 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

8. The expression system of claim 1, wherein the Drosophila cells are Drosophila Schneider cells.

9. The expression system of claim 2, wherein the Drosophila cells are Drosophila Schneider cells.

10. The method of claim 3, wherein the Drosophila cells are Drosophila Schneider cells.

11. The method of claim 4, wherein the Drosophila cells are Drosophila Schneider cells.

12. The expression system of claim 5, wherein the Drosophila cells are

Drosophila Schneider cells.

13. The method of claim 6, wherein the Drosophila cells are Drosophila Schneider cells.

14. The method of claim 7, wherein the Drosophila cells are Drosophila Schneider cells.

TI Methods of preparing carboxy-terminally truncated recombinant **flavivirus envelope** glycoproteins employing drosophila melanogaster expression systems

AI US 1997-937195 19970925 (8) <--

AB The **Flaviviridae** comprise a number of medically important pathogens that cause significant morbidity in humans including the **dengue** (DEN) virus, Japanese encephalitis (JE) virus, tick-borne **encephalitis virus** (TBE), and **yellow fever virus** (YF). **Flaviviruses** are generally transmitted to vertebrates by chronically infected mosquito or tick vectors. The viral particle which is enveloped by host cell membranes, comprises a single positive strand genomic RNA and the structural capsid (CA), **membrane** (**M**), and **envelope** (**E**) proteins. The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated and contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. Problems have arisen in the art with respect to producing recombinant forms of the **E** glycoprotein that retain their native configuration and attendant properties associated therewith (i.**e**., ability to induce neutralizing antibody responses). To date, recombinantly produced **E** glycoproteins have suffered from a number of limitations including improper glycosylation, folding, and disulfide bond formation. The claimed invention has addressed these concerns by providing secreted recombinant forms of the **E** glycoprotein that are highly immunogenic and appear to retain their native configuration. Carboxy-terminally truncated forms of **E** containing the amino terminal 395 amino acids and a suitable secretion **signal sequence** were generated in Drosophila melanogaster Schneider cell lines. The recombinant proteins produced by this expression system should prove useful, inter. . .

SUMM The invention relates to protection against and diagnosis of **dengue** fever. More specifically, the invention concerns a subunit of the **dengue** virus **envelope** protein secreted as a mature recombinantly produced protein from eucaryotic cells which is protective against **dengue** infection, which raises antibodies useful in passive immunization, and which is useful in diagnosis of infection by the virus.

SUMM The **dengue** viruses are members of the family **Flaviviridae** which also includes the Japanese encephalitis (JE) virus, Tick-borne encephalitis (TBE) virus, and the is initially discovered prototype of this class, the yellow fever (YF) virus. The **flaviviruses** contain a single positive strand genomic RNA and are small enveloped viruses affecting animals, but generally transmitted to vertebrates by chronically infected mosquito or tick vectors. **Flaviviruses** are enveloped by host cell **membrane** and contain the three structural proteins capsid (C), **membrane** (**M**), and **envelope** (**E**). The **E** and **M** proteins are found on the surface of the virion where they are anchored in the **membrane**. Mature **E** is glycosylated, whereas **M** is not, although its precursor, preM, is a glycoprotein. Glycoprotein **E**, the largest structural protein, contains functional domains responsible for cell surface attachment and intraendosomal fusion activities. It is also a. . .

SUMM **Dengue** virus is the causative agent of **dengue** fever and is transmitted to man by Aedes mosquitoes, principally Aedes aegypti and Aedes albopictus. Classic **dengue** fever is an acute illness marked by fever, headache, aching muscles and joints, and rash. A fraction of cases, typically in children, results in more extreme forms of infection, i.**e**., **dengue** hemorrhagic fever (DHF) or **dengue** shock syndrome (DSS). Without diagnosis and prompt medical intervention, the

sudden onset and rapid progress of DHF/DSS can be fatal.

SUMM **Dengue** is one of the most important virus groups transmitted to man by arthropods in terms of global morbidity; it has been estimated that **dengue** is responsible for up to 100 million illnesses annually. With the advent of modern jet travel, **dengue** has spread globally in the tropics and subtropics, and multiple **dengue** serotypes in a region are common.

SUMM Every **flavivirus** genome is a single positive-stranded RNA of approximately 10,500 nucleotides containing short 5' and 3' untranslated regions, a single long. . . products encoded by the single, long open reading frame are contained in a polyprotein organized in the order, C (capsid), preM/**M** (**membrane**), **E** (**envelope**), NS1 (nonstructural), NS2a, NS2b, NS3, NS4a, NS4b, and NS5 (Chambers, T. J. et al. Ann Rev Microbiol (1990) 44:649-688). Processing. . . and the amino terminal sequences of the viral proteins. Subsequent to initial processing of the polyprotein, preM is converted to **M** during virus release (Wengler, G. et al. J Virol (1989) 63:2521-2526), and anchored C is processed during virus maturation (Nowak. . .

SUMM There are four antigenically related **dengue** viruses which, however, can be recognized as distinct serotypes. The complete genomic sequence for at least one strain of each of the four **dengue** serotypes has been reported (DEN-1, Fu, J. et al. Virology (1992) 188:953-958; DEN-2, Deubel, V. et al. Virology (1986) 155:365-377;. . . 162:167-180; DEN-3, Osatomi, K. et al. Virus Genes (1988) 2:99-108; Osatomi, K. et al. Virology (1990) 176:643-647; DEN-4, Zhao, B. **E**. et al. Virology (1986) 155:77-88; Mackow, **E**. et al. Virology (1987) 159:217-228). In addition, the compete genomic sequences of other **flaviviruses** are known (**e**.g., YF virus: Rice et al., Science (1985) 229:726-733).

SUMM It does not appear that infection by one **dengue** serotype can confer long-term immunity on the individual with respect to other serotypes. In fact, secondary infections with heterologous serotypes. . . antibodies are directed against type-specific determinants. On the other hand, secondary infections by heterologous serotypes generate IgG antibodies which are **flavivirus** crossreactive.

SUMM Helpful reviews of the nature of the **dengue** disease, the history of attempts to develop suitable vaccines, and structural features of **flaviviruses** in general as well as the molecular structural features of the **envelope** protein of **flaviviruses** are found in Halstead, S. B. Science (1988) 239:476-481; Brandt, W. **E**. J Infect Disease (1990) 162:577-583; Chambers, T. J. et al. Annual Rev Microbiol (1990) 44:649-688; Mandl, C. W. et al. Virology (1989) 63:564-571; and Henchal, **E**. A. and J. R. Putnak, Clin Microbiol Rev (1990) 3:376-396.

SUMM A successful vaccine for protection against **dengue** infection has never been developed. However, there have been a number of preliminary efforts, many of which focus on the **envelope** protein, since this protein is exposed at the surface and is believed to be responsible for eliciting immunity.

SUMM Monoclonal antibodies (Mabs) directed against purified **E** of several **flaviviruses** DEN-2 (Henchal et al. Am J Trop Med Hyg (1985) 34:162-169, TBE (Heinz, F. X. et al. Virology (1983) 126:525-537),. . .

SUMM Although the primary amino acid sequence of the **flavivirus E** glycoprotein is variable (45-80% identity), all have twelve conserved cysteine residues, forming six disulfide bridges, and hydrophilicity profiles are nearly. . . antibody competitive binding studies, monoclonal antibody binding to purified proteolytic fragments, and analysis of neutralizing antibody escape mutants of Tick-Borne **Encephalitis Virus**, glycoprotein **E** was divided into three antigenic domains (A, B, and C) and two transmembrane segments at its carboxy-terminus. See, for example,. . .

SUMM . . . 200-250 containing five of the six disulfide bridges. Neutralization and hemagglutination inhibition epitopes are found within domain A, and, for **dengue** viruses, one of the two N-linked glycosylation sites. A conserved hydrophobic amino acid sequence within domain A has been postulated to provide fusogenic properties after low pH treatment. Amino acid sequences conserved among the **flavivirus**

family are located within this region; thus, broadly **flavivirus**-cross-reactive epitopes lie within this domain.

SUMM . . . was identified as a continuous domain composed of amino acids 301-395 (an approximate region between amino acids 300-400 for all **flaviviruses**). The domain can be isolated as a single immunoreactive proteolytic fragment. It has been postulated that this domain forms part. . .

SUMM Many strategies are currently under investigation to develop an effective and safe **dengue** vaccine; however, to date, no single strategy has proven completely satisfactory. Attempts to generate live attenuated **dengue** vaccine strains have not been entirely successful, although research into this area continues. In the absence of effective, live attenuated **dengue** vaccines, a significant effort has been invested in the development of recombinant, **dengue** subunit or viral-vectored vaccines.

SUMM Recombinant **dengue** proteins have been expressed in several systems to date (see Putnak, R. A. (1994) Modern Vaccinology, **E**. Kurstak ed., Plenum Medical, New York, pp. 231-252, for review). Most efforts using Escherichia coli have yielded poor immunogen unable to elicit neutralizing antibodies. This may reflect non-native conformation of **dengue** proteins expressed in the bacteria and the necessity to process the viral proteins through the secretion pathway in order to. . .

SUMM Several reports have described vaccinia-**flavivirus** recombinants expressing **envelope** protein as part of a polyprotein (**e**.g. C-preM-**E**-NS1; [**Dengue**] Zhao, B. G. et al. J Virol (1987) 61:4019-4022; Deubel, V. et al. J Gen Virol (1988) 69:1921-1929; Bray, **M**. et al. J Virol (1991) 63:2853-2856; [YF] Hahn, Y. S. et al. Arch Virol (1990) 115:251-265), as a single protein (**e**.g. 100% **E**; [**Dengue**] Bray, **M**. et al., J Virol (1989) 63:2853-2856), or as polypeptides (**e**.g. 79% **E**-RKG; Men, R. et al. J Virol (1991) 65:1400-1407). The most successful recombinant vaccinia viruses, those capable of inducing neutralizing antibodies and protecting mice from virus challenge, were the which were secreted **E** extracellularly or accumulated **E** on the cell surface.

SUMM Men, R. et al. (1991, supra) described the recombinant production of various C-terminal truncations of the DEN-4 **envelope** protein using a recombinant Vaccinia virus vector and infecting mammalian CV1 cells. The results showed that the recombinants that contain greater than 79% of the coding sequence produced an intracellular protein that could be immunoprecipitated with anti-**dengue** virus antibodies contained in hyperimmune mouse ascitic fluid (HMAF). Although there was a reduced level of detection for protein which contained 79% of **envelope** or less, this did not appear to result from reduced production of the protein. It was also found that only truncations which contained 79% of **E** or less were secreted efficiently; **E** polypeptides equal to or larger than 81% **E** were not secreted efficiently.

SUMM Men et al. (1991, supra) constructed additional C-terminal truncations between 79% **E** and 81% **E** to map the amino acids responsible for the difference in secretion and immunoreactivity with HMAF of these two truncated **E** polypeptides. The results demonstrated that 79% **E** containing the additional tripeptide sequence RKG was also secreted. Although both 59% **E** and 79% **E**-RKG were secreted, only 79% **E**-RKG was detected at the cells' surface. The recombinant Vaccinia viruses containing various truncations were also used to immunize mice. Mice immunized with recombinants expressing 79% **E**-RKG or larger portions of the **envelope** protein were protected. However, except for 59% **E**, mice immunized with 79% **E** or a smaller product were only partially protected. The 59% **E** elicited high protection rates (>90%) comparable to 79% **E**-RKG and larger C-terminal truncated **E** polypeptides. Protection correlated with binding to HMAF.

SUMM . . . Putnak, R. A. et al. Am J Trop Med Hyg (1991) 45:159-167; Deubel, V. et al. Virology (1991) 180:442-447). Baculovirus-expressed **dengue** and JE **E** glycoprotein elicited neutralizing antibodies, protected mice from a lethal **dengue** virus challenge, or both. In spite of these successes, the expression levels reported in baculovirus are low and the recombinant. . .

SUMM Research with purified polypeptides released by proteolysis of **flavivirus envelope** proteins, with recombinant polypeptides, and with synthetic peptides has indicated where protective epitopes may map. The isolated 9000 dalton domain. . .

SUMM A cyanogen bromide-cleaved 8 kD fragment (amino acids 375-456) overlapping domain B from JE **envelope** protein was found to induce neutralizing antibodies in mice (Srivastava, A. K. et al. Acta Virol (1990) 34:228-238). Immunization of mice with a larger polypeptide (JE **E** amino acid 319 to NS1 amino acid 65) spanning the 8 kD peptide expressed in Escherichia coli as a fusion. . .

SUMM Mason, P. W. et al. J Gen Virol (1990) 71:2107-2114 identified two domains of the DEN-1 **envelope** protein: domain I which includes amino acids 76-93 of the **E** protein and domain II (equivalent to domain B) which includes amino acids 293-402. These domains were identified from deletion analysis using recombinant fusion proteins expressed in **E**. coli and reacted with antiviral monoclonal antibodies. Recombinant fusion proteins containing **E**. coli trpE sequences fused to the **envelope** protein (amino acids 1 to 412) elicited antibodies in mice which reacted with a portion of the protein containing domain. . .

SUMM In addition, Mason, P. W. et al. (J Gen Virol (1989) 70:2037-2049) expressed a collection of **E**. coli trpE fusion proteins to segments of JE virus **envelope** protein spanning domain B. The trpE fusion proteins containing the smallest JE **E** fragments that retained immunoreactivity with a panel of neutralizing monoclonal antibodies included amino -acid residues from methionine 303 through tryptophan. . .

SUMM Trirawatanapong, T. et al. Gene (1992) 116:139-150. prepared several truncated forms of **dengue** 2 **envelope** proteins in **E**. coli for epitope mapping, and mapped monoclonal antibody 3H5 to its corresponding epitope. This was first localized between amino acids. . . amino acids between positions 386 and 397. The mapping was apparently confirmed by the ability of a synthetic peptide containing **E** protein amino acids 386-397 to bind 3H5 specifically.

SUMM Megret, F. et 41. Virology (1992) 187:480-491 prepared 16 overlapping fragments of DEN-2 **envelope** protein as trpE fusion products in **E**. coli for epitope mapping. The fusion proteins are produced intracellularly and obtained from the lysates. These products were used to. . .

SUMM . . . findings of Trirawatanapong et al. Gene (1992, supra), MAb 3H5 was unable to bind to trpE fusion proteins containing DEN-2 **E** amino acids 304-397, 298-385, or 366-424. The two exceptional MAbs in the findings of Megret et al. are MAbs 5A2. . .

SUMM Although it appears established from the art that the B domain of the **flavivirus envelope** protein contains epitopes which bind neutralizing antibodies, problems have arisen with respect to producing recombinant polypeptides containing the B domain. . . a form which mimics the native protein and is thus capable of eliciting an immune response. The only recombinantly produced **E** polypeptides containing the B domain that elicited a protective immune response in mice were expressed from Vaccinia and baculovirus vectors.. . . extensions do not interfere with the immunogenic effectiveness or secretion of the B domain. In one embodiment, such extensions are minimal--i.**e**., not more than six additional amino acids--at either the N-terminus or the C-terminus, or distributed between these termini; preferably no. . .

SUMM . . . domain includes at least portions of the region extending to amino acid 413, the additional region may confer additional functions, **e**.g., enhancing immunogenicity by providing a helper T cell epitope. The form of domain B which includes positions about 296-413 is. . .

SUMM Other portions of the **E** protein illustrated below are self-explanatory. 80% **E** is the N-terminal 80% of the protein from residue 1 to residue 395. 60% **E** represents the corresponding shorter sequence. These subunits are produced from vectors containing the DNA encoding the mature protein, or along with the **prM** fusion which results in secretion of the 80% or 60% per se.

SUMM It has now been found that the B domain of the **envelope** protein can be successfully secreted from yeast in a form which elicits the production of neutralizing antibodies. This permits, for the first time, the

production of a successful recombinantly produced subunit **dengue** vaccine.

SUMM The invention provides vaccines containing as an active ingredient, a secreted recombinantly produced the **dengue envelope** protein or a subunit thereof. The vaccines are capable of eliciting the production of neutralizing antibodies against-**dengue** virus. In the illustrations below, the B domain of the **envelope** protein (**E**) is secreted from yeast by producing it in an expression vector containing the α-mating factor prepropeptide leader sequence (preproMFα_L). Peptide subunits representing 60% **E** and 80% **E** are secreted from Drosophila cells using the human tissue plasminogen activator secretion **signal sequence** for the propeptide (tPA$_L$) or from the homologous **premembrane** (**prM**) leader. The secreted products can easily be purified and prepared as a vaccine.

SUMM Thus, in one aspect, the invention is directed to a vaccine for protection of a subject against infection by **dengue** virus. The vaccine contains, as active ingredient, the **envelope** protein of a **dengue** virus serotype or a subunit thereof. The **E** or subunit is secreted as a recombinantly produced protein from eucaryotic cells. The vaccine may further contain portions of additional **dengue** virus serotype **E** proteins similarly produced.

DRWD FIG. 1 is a drawing reproduced from Mandl, et al. (supra) showing a model of the **envelope** protein of **flaviviruses**. Model of the TBE virus protein **E**. Open circles represent hydrophilic amino acid residues (Arg, Lys, Asn, Asp, Gln, Glu, His), dotted circles show intermediate amino acid. . . Small arrows indicate potential cleavage sites within these fragments that are not utilized. Two solid lines stand for the lipid **membrane** that is spanned by two transmembrane regions of protein **E**. The polypeptide chain is folded to indicate the antigenic domains A, B, and C, which are designated by large capital. . . solid diamond represents the carbohydrate side chain of TBE virus. The Murray Valley (MVE), St. Louis (SLE) and Japanese (JE) **encephalitis viruses** and DEN viruses have potential N-glycosylation sites at the homologous position. Yellow fever (YE) and St. Louis **encephalitis viruses** have such a site within domain B. DEN viruses within domain A. The homologous positions of TBE virus are shown. . .

DRWD . . . strain and differences from the wild-type strain reported by Hahn (1988, supra). DEN-2 PR159/S1 cDNA coding strand sequence for Capsid, **preMembrane**, **Envelope**, and NS1 is given (Hahn etr al. 1988). The start of genes are indicated. "*" indicates correction to published sequence.. . .

DRWD . . . of SEQ ID NO:1 and SEQ ID NO:4 through SEQ ID NO:7) shows the oligonucleotide used to mutagenize an 80% **E** cDNA clone to obtain the domain B coding sequence. Shown are the necleotide sequence and the corresponding translation of DEN-2 PR159/S1 **E** sequences between genomic nucleotides 1789 and 1848, the sequence of the oligonucleotide used in mutagenesis, and the resulting sequence and corresponding translations following mutagenesis. **Dengue** nucleotide sequence is uppercase, non-**dengue** nucleotides are longer case, inserted restriction endonuclease sites are indicated, and conserved cysteines are underlined.

DRWD FIG. 10 shows the survival times of mice immunized with recombinant domain B and challenged with **Dengue**-2.

DETD . . . subunit vaccine that can be efficiently produced recombinantly and secreted and that is effective in protecting subjects against infection with **dengue** virus. Although many attempts have been made to obtain such a subunit vaccine, either the subunit itself is resistant to. . . recombinant production is facile, it fails to elicit neutralizing antibodies. The present inventors have found that certain portions of the **envelope** protein of **dengue** virus type 2, such as domain B representing approximately 100 amino acids of the **envelope** protein extending approximately from the Gly at position 296 to the Gly at position 395, and optionally including additional **E** sequence through position 413 of the protein, and other portions of **E**, i.**e**., 60% **E** and 80% **E** are effectively secreted by certain convenient eucaryotic recombinant hosts, in a form that permits processing to mimic

the native conformation. . . . to raise neutralizing antibodies in animals. Thus, this subunit represents a useful component of a vaccine for protecting subjects against **dengue** infection.

DETD . . . used herein, "B domain" refers to a peptide which spans from approximately Gly 296 to Gly 395 of the DEN-2 **envelope** protein, and optionally including additional **E** sequence through position 413 of the **envelope** protein. These positions are approximate; for example, Mandl (1989, supra) describes the generation of a tryptic fragment containing domain B which spans the amino acids of the TBE **E** protein from position 301 to 396. The sequences described in the present application represent the **envelope** protein from **dengue** Type 2 virus (DEN-2); three additional distinct **dengue** serotypes have been recognized. Therefore, "Domain B" also refers to the corresponding peptide region of the **envelope** protein of these serotypes, and to any naturally occurring variants. In addition, B domain includes extended forms of the about. . . the subunits as produced must assume a conformation and undergo processing under conditions which, render them similar to the native **envelope** portion as it exists in the **envelope** protein of the virus. In order to achieve this, the recombinant production must be conducted in eucaryotic cells, preferably yeast. . . ovary cells. Other insect cells may also be used in conjunction with baculovirus based vectors. The B domain or 60% **E** or 80% **E** must be produced as a correctly processed protein and secreted.

DETD . . . ways. First, this can be done by expressing the B domain in yeast in operable linkage with the α-mating factor **signal sequence**. Constructs which place the nucleotide sequence encoding the B domain disposed so as to encode a fusion protein with an upstream α-mating factor **signal sequence** are therefore included within the scope of the invention. An additional preferred embodiment employs Drosophila cells and the human tissue plasminogen activator leader sequence for secretion of 60% **E** or 80% **E** as well as domain B. **Envelope** protein subunits that represent N-terminal portions of truncated protein may also be secreted from the homologous **prM** fusion. Other secretion signal peptides or secretion leader pre/pro peptides, such as those associated with invertase or acid phosphatase of. . . In general, the invention includes expression systems that are operable in eucaryotic cells and which result in the formation of **envelope** protein or a subunit secreted into the medium. Thus, useful in the invention are cells and cell cultures which contain. . .

DETD The properly processed **E** protein or subunit is recovered from the cell culture medium, purified, and formulated into vaccines. Purification and vaccine formulation employ. . .

DETD To immunize subjects against **dengue** fever, the vaccines containing the subunit are administered to the subject in conventional immunization protocols involving, usually, multiple administrations of. . .

DETD . . . can themselves be used as passive vaccines. For production of passive vaccine, a suitable mammalian subject is immunized with the **E** protein or subunit of the invention and antibodies are either recovered directly as a composition from the antisera or indirectly. . .

DETD In addition to use in vaccines or in the generation of passive vaccines, the mature recombinant **E** protein and subunits of the invention may be used as analytical reagents in assessing the presence or absence of antidengue antibodies in samples. The interest in doing this may be diagnosis of infection with **dengue**, monitoring response to **dengue** infection or may simply reside in the use of immunoassays as part of standard laboratory procedures in the study of. . .

DETD Thus, the secreted protein, such as 60% **E**, 80% **E** or B domain may be adsorbed onto solid support and the support then treated with a sample to be tested. . .

DETD In addition, both the mature peptides, such as domain B and 60% **E** or 80% **E** of the invention and the antibodies immunoreactive with it can be used in standard purification procedures as affinity reagents. Thus,. . .

DETD In the examples below, particular subunits of the **dengue** Type 2 **envelope** protein, in particular 60% **E**, 80% **E** and domain B are illustrated as representative of effective subunits of the **envelope**

protein. For the 80% **E** and 80% **E** constructs in general, secretion can be obtained from constructions designed to express the prME subunit fusion. The mature N-terminus of the **envelope** protein is then secreted into the culture medium. Whether the N-terminus of the **envelope** protein subunits were fused to a heterologous leader, such as the human tissue plasminogen activator leader sequence, or to the homologous **prM** sequence, the mature form of the truncated **envelope** protein is secreted. The secreted truncated Es are expressed at high levels in Drosophila, efficiently processed, and secreted into the medium. The products are glycosylated and processed to an endo-H resistant form. The secreted form of truncated **E** produced cotranslationally with **prM** generally represents about 20-30% of the total protein in the medium. Furthermore, based upon reactivity with conformationally sensitive monoclonal antibodies, using a ELISA and immunofluorescence formats, the secreted **E** products are shown to have a native conformation. Immunization of mice with crude medium from transformed cells expressing **prM**-truncated **E** induces a potent virus-neutralizing response.

DETD Preparation of **Envelope** Proteins in Saccharoimyces cerevisiae

DETD A cDNA clone derived from **dengue** serotype 2 (DEN-2) described by Hahn, Y. S. et al. Virology (1988, supra) was used as the starting material. This. . .

DETD FIG. 2 shows the sequence of the CDNA derived from genomic RNA of DEN-2 PR159/S1 for the Capsid, **preMembrane**, **Envelope**, and NS1 genes. Shown in bold at nucleotides 103, 1940, 1991, and 2025 are corrections to the Hahn published sequence.. . . sequence from the wild-type sequence are noted above the wild-type sequence. There are no nucleotide differences in the Capsid and **preMembrane** protein-encoding portions and there are four in the **E** encoding portion.

DETD FIG. 3 shows the cDNA sequence of DEN-2 PR159/S1 for the Capsid, **preMembrane**, **Envelope**, and NS1 genes and the inferred translation of those four genes, which is part of the larger **dengue** polyprotein. The four differences between wild-type DEN-2 PR159 and the S1 strain are shown above the S1 nucleotide sequence. Also. . .

DETD In the **E** gene, three of the four mutations are silent; S1 has G instead of A at position 1314, T rather than. . . conservative, mutagenesis studies of other viral structural proteins (Coller, B. G. et al. (1994) Mutagenesis Studies on the Predicted VP2 **E**-F Loop of Coxsackievirus B3, Abstract, 13th Annual Meeting of the American Society for Virology) have demonstrated that even relatively conservative. . .

DETD Various **E** gene subclones were obtained which represented the amino-terminal 90% of the **envelope**, 80% of the **envelope**, 60% of the **envelope** and classical domain B. Using the assignment of Mandl, C. W. et al. J Virol (1989) 63:564-571, classical domain B. . . et al. at its carboxy end which can be included in some forms of the domain B of the invention, **e**.g., DomB+T.

DETD The portion of the genome that encodes 80% of the **envelope** protein (80% **E**) was amplified using the Polymerase Chain Reaction, primers D2E937p and D2E2121m, and plasmid pC8 (Hahn et al. (1988, supra) as. . .

DETD In this notation of the primers, the virus serotype is first indicated (D2 for DEN-2), then the corresponding **dengue** gene--i.**e**., in this case **envelope**, **E**, is noted. Then is noted the number in the **dengue** cloned sequences of FIGS. 2 or 3 for the first **dengue** nucleotide in the 5'-3' direction of the oligonucleotide, i.**e**., using the numbering of Hahn et al. (1988, supra), and finally the notation shows whether the oligonucleotide primes the plus (p) or the minus (**m**) strand synthesis. The sequence in the primers corresponding to **dengue** cDNA are written in uppercase letters; nondengue sequence is written in lowercase letters. D2E937p2 - BglII 5'-cttctagatctcgagtacccgggacc ATG CGC TGC. . .

DETD The D2E2121m primer placed two stop codons after the 395th codon of **E**. The 80% **E** amplified cDNA fragment was digested at the XbaI sites in the cloning adapters and cloned into the NheI site of pBR322 to obtain p29D280E. Double-strand sequence for 80% **E** was determined, which identified a single silent PCR-introduced mutation at nucleotide 2001 (AAC/Asn to AAT/Asn).

DETD A subclone representing domain B was obtained from the 80% **E** subclone by oligonucleotide-directed mutagenesis. In the mutagenesis, stop codons and restriction endonuclease sites were inserted between domain C- and domain. . . FIG. 4, to avoid a high AT content in the mutagenic oligonucleotide, the stop codons defining the carboxy-terminus of 60% **E** containing domains A and C were positioned four codons upstream of the beginning of domain B, i.**e.**, following Lys291. The original and altered nucleotide sequences of the mutagenized region and the corresponding amino acid translation are shown. . .

DETD To perform the mutagenesis, a 580 bp BamHI fragment spanning domain B from the pBR322-80% **E** clone p29D280E was subcloned into pGEM3Zf (Promega) to yield p29GEB2. (See FIG. 5.) This BamHI fragment encodes the 3' end. . .

DETD The cloned cDNA fragments encoding B domain and 80% **E** were inserted into expression vectors so as to maintain the translational frame of fusions to secretion leaders as described below.. . .

DETD The expression vector constructed to secrete classical domain B from Saccharomyces cerevisiae to include **envelope** protein amino acids 296-395 was constructed so that processing by the proteases normally involved in preproMFα_L processing would yield a. . .

DETD . . . cloning sites, and use the TRP1 gene as a selectable marker. They contain sequences derived from pBR322 to provide an **E**. coli origin of replication, the ampicillin resistance gene, and sequences derived from the 2-micron plasmid of S. cerevisiae to enable. . .

DETD . . . or Glu-Ala-DomB. For this demonstration, proteins secreted by a pLS5-DomB transformant were separated by SDS-PAGE and electroblotted to Immobilon P **membrane** (Millipore), and the amino terminal amino acid sequence was determined by microsequencing. That sequence is: H_2 N-Glu Ala Gly Met. . .

DETD . . . sodium phosphate (pH 6.7), the cells were pelleted by centrifugation and the medium was clarified by filtration through a 0.45 **μm** pore filter. The filtered medium was concentrated about 30-fold either by tangential flow or by centrifugal ultrafiltration using Minitan (Millipore). . .

DETD . . . color and a mostly colorless flow-through containing domain B. For DEAE chromatography, the brown-colored pooled fractions were dialyzed against 0.1 **M** acetic acid, pH 5.1 and loaded onto a 1.4×15 cm DEAE (Biorad) column. Domain B was eluted using a 0.01 **M** acetic acid, pH 5.1, 0.1 **M** NaCl step gradient.

DETD . . . domain B by overnight incubation at 4° C. and then blocked with BSA in 50 mM tris-HCl, pH 7.0, 0.15 **M** NaCl, 0.05% Tween for 1 hour at room temperature. The plates were then treated with either DEN-2 HMAF or monoclonal. . .

DETD . . . of the secreted yeast proteins and the second as 70-90% pure domain B. Treatment with 1M NaCl, 1 and 2 **M** urea, and 1% DDAPS, a zwitterionic detergent, were ineffective in completely disaggregating domain B during size exclusion chromatography. Since the. . .

DETD . . . 24 hours, the culture is supplemented with 0.01 vol of sterile 40% w/v glucose and 0.02 vol of sterile 1 **M** phosphate buffer, pH 6.7.

DETD . . . final concentrations of 1 mM each to the cleared medium, and the resulting solution is filter sterilized using a 045 **μm** pore filter **membrane** (Millipack-20 or Opticap-50, Millipore). Glycerol is added to 10% v/v to the filtrate which is then concentrated 20-30 fold using tangential flow ultrafiltration (Millipore Minitan System) with two **membrane** cartridges (four regenerated cellulose membranes) of a 10 kD MW cutoff. The retentate is kept on ice during ultrafiltration in. .

DETD Concentrated medium of the tertiary culture is dialyzed at 4° C. using a **membrane** with a 6-8 kD cutoff (Spectra/Pore-1 **Membrane**) against 10 mM acetate, pH 4.5 (4×4 liters; 2-3 hours each; 1 overnight).

DETD The domain B-containing fractions are pooled and concentrated about 11-fold by centrifugal ultrafiltration using, for example, a Centriprep-10® (brand **membrane** ultrafiltration system) (Amicon) and loaded onto a 5×60 cm Sephadex G-75® (brand polysepharose)

(Superfine, Pharmacia) column at 4° C. The. . .

DETD . . . containing pure immunoreactive domain B from 2-4 purification runs are concentrated about 50-fold by centrifugal ultrafiltration using a Centriprep-10® (brand **membrane** ultrafiltration system) (Amicon; final volume 2-5 ml). The pooled material is stored at 4° C.

DETD . . . replacing the glycerol) and cultured 48 hrs. Culture media with cells removed by sequential centrifugation and filtration through a 0.45 μm pore size **membrane** were buffer exchanged by diafiltration into TEEN (10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM EGTA, and 150. . .

DETD . . . a fusion with the $MF\alpha_L$. This construct includes those sequences that lie between domain B and the transmembrane anchor of **E**. This region contains a potential T cell epitope (Mandl et al. J Virol (1989) 63:564-571) and additional hydrophobic sequences, a. . .

DETD The domain B+stem cDNA fragment was constructed in **E**. coli cloning vectors by combining the domain B cDNA fragment and the 3' end of a 90% **E** clone. As introduced in Example 1, an **E** gene subclone representing the amino terminal 90% of **E** was constructed from DEN-2 PR159/S1 cDNA plasmid pC8 of Hahn et al. (1988, supra) using the PCR. The 90% **E** polypeptide contains all of **E** except for the C-terminal **membrane** anchor comprising two transmembrane domains. The 90% **E** cDNA clone was made as follows. The 90% **E** fragment was amplified by the PCR using pC8 as template and primers D2E937p and D2E2271m. The sequence of D2E937p is. . .

DETD . . . of #, and the two primers positioned useful restriction enzyme sites at both ends of the fragment. The PCR-amplified 90% **E** cDNA fragment was made blunt at both ends and cloned into the SmaI site of a modified pUC13 cloning vector,. . .

DETD Combining domain B and the 90% **E** 3' end made use of a unique Af1III restriction enzyme site found in most pUC-like cloning vectors and a unique Af1III site in domain B sequences. This combining was accomplished by first subcloning the 90% **E** fragment from pVZ90E into pBluescript to reverse the orientation of 90% **E** relative to the vector sequences, yielding pBS90E. Then, p29GEB24PS, containing domain B sequences in PGEM (Example 1), and pBS90E were. . . Af1III, and the vector-domain-B5' fragment and the domain-B3'-stem-vector fragment from the two digestions, respectively, were purified, ligated, and recovered in **E**. coli yielding pBS-Bstem.

DETD . . . yielded high-titre antibodies that were highly immunoreactive when used to probe Western blots displaying the same antigen or DEN-2 viral **envelope** protein.

DETD The serological diagnosis for **dengue** infection is based on the detection of anti-**dengue** IgM and IgG in primary and secondary viral infection using standard Enzyme Linked Immunosorbent Assay (ELISA) techniques. Current assays are based on the ability of anti-**dengue** immunoglobulins to recognize semi-purified virus. Primary and secondary infections can be distinguished by the IgM:IgG ratios. (Innis et al., 1989;. . .

DETD . . . in both IgM and IgG tests. In these ELISAs, the antigen was coated on plates, followed by sera positive for **dengue** antibodies and then detection by goat antihuman antibody.

DETD . . . with 100 μl of a 5 μg/ml solution of domain B. After blocking with 3% normal goat serum, primary infected **dengue** 2 positive serum at a 1:100 dilution was added per well. The bound IgM was detected by the addition of. . .

DETD For IgG, high titer secondary **dengue** 2 infected sera were supplied at a 1:270 dilution and detected with goat anti-human IgG (Fc) conjugated to horseradish peroxidase.. . .

DETD . . . purified by the improved procedure of Example 3 is not recognized by murine polyclonal hyperimmune ascitic fluid (HMAF) to other **dengue** serotypes and to other **flaviviruses**, when assayed using a similar ELISA format. **Flavivirus** infected murine sera tested include, Japanese **Encephalitis virus**, Tick-Borne **Encephalitis virus, Yellow Fever virus**, Saint Louis **Encephalitis virus**, West Nile virus, three viral isolates of **dengue** serotype 1, two viral isolates of **dengue** serotype 3, and two viral isolates of **dengue** serotype 4.

DETD Sandwich assay for the detection of any domain B containing **envelope** antigen.

DETD In an alternative enzyme immunoassay format, anti-domain B or anti-**Dengue** capture antibody, polyclonal or monoclonal, may be absorbed to the solid support, and sample containing an unknown quantity or serotype of **dengue** antigen may be added and then detected by reacting with a second anti-domain B or anti-**Dengue** antibody, either conjugated to a signal-generating enzyme or to be detected using a appropriate signal generating system, of which there are a multitude. This immunoassay is useful for the quantitation of recombinantly produced **envelope** protein or whole virus by comparing the immunoreactivity of a known concentration of domain B with that of the unknown. . . epitope binding site of the anti-domain B capture antibody can be varied to garner additional information regarding the conformation of **dengue** protein in the preparation.

DETD To perform the sandwich enzyme immunoassay, 100 µl of anti-**Dengue** monoclonal antibody 9D12 or 3H5 (Henchal, **E**. A. et al., Am J Trop Med Hyg (1985) 34:162-169) was used to coat microtiter wells. The monoclonal antibodies were. . . by Protein-A affinity chromatography and used at 10 µg/ml concentration (diluted in PBS: 50 mM sodium Phosphate, pH 7.0, 0.15 **M** NaCl). After a one hour incubation, the wells were washed three times with TBS-T (50 mM Tris-HCl PH 7.0, 0.15 **M** NaCl, 0.05% Tween-20), and blocked with 200 µl/well of 1% BSA in PBS for 1 hour at room temperature. Following. . . 200 µl/well of 1 mg/ml p-nitrophenylphosphate (pNPP) substrate in alkaline phosphatase substrate buffer (25 mM Trizma base, pH 9.5, 0.15 **M** NaCl, 5 mM $MgCl_2$; 0.02% NaN_2) was added. The plates were incubated for one hour at room temperature and. . .

DETD . . . an intracerebral injection of DEN-2 New Guinea C (NGC) strain. DomB administered in all adjuvants conferred comparable moderate survival against **dengue** virus challenges although survival was statistically significant (P<0.5 G test) only for mice immunized with DomB and Hunter's TiterMax. The. . .

DETD **E**. DomB Immunizations for Hybridoma Generation:

DETD Cells cotransfected at ratios of 1:1, 5:1 and 20:1 were induced with 200 **µM** copper sulfate and the media and cells were harvested at days 1, 4 and 7. Western blots showed secretion of. . .

DETD Production of 60% **E** and 80% **E** in Drosophila

DETD . . . Drosophila metalothionein gene, the human tissue plasminogen activator signal and the SV-40 early polyadenylation signal, the nucleotide sequences encoding 80% **E**, **prM** 80% **E**, 60% **E** and **prM** 60% **E** are inserted and the resulting vectors used to transform Schneider cells as described in Example 9. The mature truncated forms of the **envelope** protein are secreted into the medium, or properly processed, and are conformationally correct with respect to the corresponding native portions of the **envelope** protein.

DETD . . . employed in the protocol set forth in paragraphs A and B of Example 8 produce antibodies which are neutralizing against **dengue** virus.

DETD . . . acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: cDNA
- - (vi) ORIGINAL SOURCE:
(A) ORGANISM: **Dengue** vi - #rus
(B) STRAIN: Serotype 2 - #(Den-2)
- - (vii) IMMEDIATE SOURCE:
(B) CLONE: Den-2 PR159/ - #S1
. . . (A) NAME/KEY: misc__ - #feature
(B) LOCATION: 343
(D) OTHER INFORMATION: - #/note= "Start of coding strand sequence - #for **preMembrane**"
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 616

```
          (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #for Membrane"
  - -      (ix) FEATURE:
           (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 841
           (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #for Envelope"
  - -      (ix) FEATURE:
           (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 2326
           (D) OTHER INFORMATION: - #/note= "Start of coding.  .  .  acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear
  - -      (ii) MOLECULE TYPE: cDNA
  - -      (vi) ORIGINAL SOURCE:
           (A) ORGANISM: Dengue vi - #rus
           (B) STRAIN: Serotype 2( - #DEN-2)
  - -     (vii) IMMEDIATE SOURCE:
           (B) CLONE: Den-2 PR159/ - #S1
.  .  .   (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 343
           (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #for preMembrane"
  - -      (ix) FEATURE:
           (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 616
           (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #of Membrane"
  - -      (ix) FEATURE:
           (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 841
           (D) OTHER INFORMATION: - #/note= "Start of coding strand
               sequence - #of Envelope"
  - -      (ix) FEATURE:
           (A) NAME/KEY: misc__ - #feature
           (B) LOCATION: 2326
           (D) OTHER INFORMATION: - #/note= "Start of coding.  .  .  - #C CCA
      CCA ACA GCA GGG
    192
Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Il - #e Pro Pro Thr Ala Gly
     50             - #     55             - #     60
 - - ATA TTA AAA AGA TGG GGA.  .  .  - #T AAG ACA AAG GAC GGC
    432
Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Ph - #e Lys Thr Lys Asp Gly
    130             - #   135             - #   140
 - - ACG AAC ATG TGT ACC CTC.  .  .
DETD              .  .  .  - #A ATG GAT CTG GAA AAA
   1872
Asp Gly Ser Pro Cys Lys Ile Pro Phe Glu Il - #e Met Asp Leu Glu Lys
    610             - #   615             - #   620
 - - AGA CAT GTT TTG GGC CGC. .  .  - #C GTC ACA GAT AAC GTG
   2400
Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Ph - #e Val Thr Asp Asn Val
785               7 - #90               7 - #95               8 -
#00
  - - CAT ACA TGG ACA.  .  .  - #A GAG AAA GCT TCT TTC
   2976
Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Il - #e Glu Lys Ala Ser Phe
           980      - #          985      - #          990
 - - ATT GAA GTC AAA AGT TGC.  .  .  - #A ACG GAA TGG TGT TGT
   3264
Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Il - #e Thr Glu Trp Cys Cys
        1075        - #      1080          - #      1085
 - - CGA TCT TGC ACA CTA CCA.  .  .  - #         40         - #         45
 - - Ala Leu Val Ala Phe Leu Arg Phe Leu Thr Il - #e Pro Pro Thr Ala Gly
```

- - Ile Leu Lys Arg Trp Gly. . . - # 120 - # 125
- - Ser Arg Gln Glu Lys Gly Lys Ser Leu Leu Ph - #e Lys Thr Lys Asp Gly
130 - # 135 - # 140
- - Thr Asn Met Cys Thr Leu. . . - # 600 - # 605
- - Asp Gly Ser Pro Cys Lys Ile Pro Phe Glu Il - #e Met Asp Leu Glu Lys
610 - # 615 - # 620
- - Arg His Val Leu Gly Arg. . . - # 775 - # 780
- - Asn Lys Glu Leu Lys Cys Gly Ser Gly Ile Ph - #e Val Thr Asp Asn Val
785 7 - #90 7 - #95 8 -
#00
- - His Thr Trp Thr. . . - # 970 - # 975
- - Ile Glu Ser Ala Leu Asn Asp Thr Trp Lys Il - #e Glu Lys Ala Ser Phe
980 - # 985 - # 990
- - Ile Glu Val Lys Ser Cys. . . - # 1065 - # 1070
- - Leu Arg Thr Thr Thr Ala Ser Gly Lys Leu Il - #e Thr Glu Trp Cys Cys
1075 - # 1080 - # 1085
- - Arg Ser Cys Thr Leu Pro. . .
DETD . . . (C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (ii) MOLECULE TYPE: peptide
- - (ix) FEATURE:
(A) NAME/KEY: Cleavage-sit - #e
#20) (B) LOCATION: (19
(D) OTHER INFORMATION: - #/note= "Signalase cleavage"
- - (ix) FEATURE:
(A) NAME/KEY: Cleavage-sit - #e
#86) (B) LOCATION: (85
(D) OTHER INFORMATION: - #/note= "Kex2p cleavage"
- - (ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION: 1..19
. . . #50 1 - #55 1 -
#60
- - Ala Glu Pro Pro Phe Gly Asp Ser Tyr Ile Il - #e Ile Gly Val Glu
Pro
165 - # 170 - # 175
- - Gly Gln Leu Lys Leu Asp Trp. . . base - #pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- - (vi) ORIGINAL SOURCE:
(A) ORGANISM: **Dengue** vi - #rus
- - (ix) FEATURE:
(A) NAME/KEY: misc__ - #feature
(B) LOCATION: 27..46
(D) OTHER INFORMATION: - #/note=. . .

1. An expression system for the recombinant production and secretion of a portion of an **envelope** (**E**) protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese **encephalitis virus** (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which expression system comprises Drosophila cells modified to contain a DNA molecule which comprises (a) a first nucleotide sequence encoding said portion of said **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395, and (b) a second nucleotide sequence which encodes a secretory leader sequence or a secretory **signal sequence** operably linked to said first nucleotide sequence and positioned so as to produce a fusion protein when said first and. . .

. . . claim 1 wherein said secretory leader sequence is human tissue plasminogen activator prepropeptide secretion leader (tPA_L) and optionally includes the **premembrane** leader of the **E** protein.

3. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese

encephalitis virus (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 1 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

4. A method to produce a portion of an **E** protein of a **Flavivirus** selected from the group consisting of **dengue** virus, Japanese **encephalitis virus** (JEV), tick-borne **encephalitis virus** (TBE) and **yellow fever virus** (YF), which method comprises (a) culturing the Drosophila cells of claim 2 in culture medium under conditions favorable for expression of the encoding nucleotide sequence so that the cells secrete said portion of the **E** protein of the **Flavivirus** strain against which protection is sought, which portion is the N-terminal 80% of the protein from residue 1 to residue 395 into the medium; and (b) recovering the portion of the **E** protein from the culture medium.

5. The expression system of claim 1 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

6. The method of claim 3 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

7. The method of claim 4 wherein the N-terminal 80% of the **E** protein from residue 1 to residue 395 is **dengue** virus **E** protein.

L14 ANSWER 7 OF 15 USPATFULL on STN
2000:74131 Recombinant **dengue** virus DNA fragment.
Kelly, Eileen P., Takoma Park, MD, United States
King, Alan D., Washington, DC, United States
The United States of America as represented by the Secretary of the Army, Washington, DC, United States (U.S. government)
US 6074865 20000613
APPLICATION: US 1995-504878 19950720 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB A recombinant protein encompassing the complete **envelope** glycoprotein and a portion of the carboxy-terminus of the **membrane/premembrane** protein of **dengue** 2 virus was expressed in baculovirus as a protein particle. The recombinant protein particle was purified and found to provide protection against lethal challenge with **dengue** 2 virus in mice.
CLM What is claimed is:
1. An isolated and purified **dengue** virus DNA fragment consisting essentially of a DNA fragment which encodes a complete **dengue** virus **envelope** protein and a carboxy terminus segment of **premembrane** protein which comprises a translocation signal for said **Envelope** protein.

2. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** virus is **dengue** 2.

3. The DNA fragment of claim 2 which encodes 495 amino acids of said **envelope** protein and 31 amino acids of said carboxy terminus segment of **premembrane** protein, said fragment comprising the nucleotide sequence specified in SEQ ID NO: 1 or an allelic variant which retains the neutralizing antibody production characteristic of a protein encoded by SEQ ID No. 1.

4. The DNA fragment according to claim 3, wherein said DNA fragment encodes the amino acid sequence specified in SEQ ID NO: 2.

5. The isolated and purified DNA fragment according to claim 1, wherein

said **dengue** is **dengue** 1.

6. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** is selected from the group consisting of **dengue** 3 and **dengue** 4.

7. A recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

8. A recombinant DNA construct according to claim 7, wherein said **dengue** virus is **dengue** 2.

9. The recombinant DNA construct according to claim 7, wherein said vector is a eukaryotic expression vector.

10. The recombinant DNA construct according to claim 8, wherein said vector is a eukaryotic expression vector.

11. A recombinant DNA construct comprising: (i) a vector, and (ii) a **dengue** 2 DNA fragment according to claim 3.

12. The recombinant DNA construct according to claim 11, wherein said vector is a eukaryotic expression vector.

13. The recombinant DNA construct according to claim 11, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO: 2.

14. The recombinant DNA construct according to claim 11 wherein said vector is pBlueBacIII.

15. A host cell transformed with a recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

16. A host cell according to claim 15, wherein said cell is prokaryotic.

17. The host cell according to claim 15, wherein said cell is a eukaryotic cell.

18. A method for producing a **dengue** virus recombinant protein particle, said method comprising the steps of: (i) culturing a host cell transformed with an expression vector according to claim 9 under conditions such that said DNA fragment is expressed and said recombinant protein is produced as a particle, said particle comprising more than one unit of said recombinant protein; and (ii) isolating said recombinant protein particle.

19. The method according to claim 18, wherein said **dengue** virus is **dengue** 2.

20. The method of claim 18 wherein isolating said recombinant protein particle comprises: (i) pelleting said cells by centrifugation, (ii) separating the cell pellet and the supernatant, (iii) lysing said cell pellet to release said recombinant protein particle; (iv) isolating said recombinant protein particle of step (iii); (v) fractionating said recombinant protein particle of step (iv) on a gradient; and (vi) isolating said recombinant protein particle, in a purified form.

TI Recombinant **dengue** virus DNA fragment

AI US 1995-504878 19950720 (8) <--

AB A recombinant protein encompassing the complete **envelope** glycoprotein and a portion of the carboxy-terminus of the **membrane/premembrane** protein of **dengue** 2 virus was expressed in baculovirus as a protein particle. The recombinant protein particle was purified and found to provide protection against lethal challenge with **dengue** 2 virus in mice.

SUMM . . . to the production and purification of a recombinant protein for

use as a diagnostic tool and as a vaccine against **Dengue** virus.

SUMM **Dengue** (DEN) viruses are human pathogens with a significant threat to world health. These viruses are estimated to cause several hundred thousand cases of **dengue** fever, **dengue** hemorrhagic fever (DHF) and **dengue** shock syndrome (DSS) annually (Shope, R. **E**. In: The Togaviruses. Schlesinger, R. W. (Ed.) Academic Press, New York. 1980, pp. 47-82; Monath, T. P. In: The Togaviridae and **Flaviviridae**, Schlesinger, S. and Schlesinger, **M**. J. (Eds.) New York and London, 1986, pp. 375-440; Halstead, S. B. Bull. W.H.O. 1980, 58, 1-21; Halstead, S. B. Am. J. Epidemiol. 1984, 114, 632-648) The complete content of all documents cited herein are hereby incorporated by reference. **Dengue** viruses are members of the family Flaviviridae and are transmitted by Aedes mosquitoes (Halstead, S. B. Science 1988, 239, 476-481).. . . neutralization tests (Russell, P. K. and Nisalak, A. J. Immunol. 1967, 99, 291-296) and immunoassays using monoclonal antibodies (MAbs) (Gentry, **M**. K. et al. Am. J. Trop. Med. Hyg. 1982, 31, 548-555; Henchal, **E**. A. et al. Am. J. Trop. Med. Hyg. 1982, 31, 830-836).

SUMM **Dengue** viruses are composed of a single-stranded RNA molecule of positive polarity (messenger sense) which is contained within a nucleocapsid composed of capsid (C) protein. The capsid is surrounded by a lipid **envelope** about 50 nm in diameter in which are embedded the **envelope** (**E**) glycoprotein and the matrix (**M**) protein. Both the structural and nonstructural (NS) proteins are encoded by a single, long open reading frame of about 10.5 kilobases arranged as follows: C-PreM/**M**-**E**-NS1-NS2A-NS2B-NS3-NS4A-NS5 (Rice, C. **M**. et al. Science 1985, 229, 726-733; Wengler, G. et al. Virology 1985, 147, 264-274; Castle, **E**. et al. Virology 1986, 149, 10-26; Zhao, B. et al. Virology 1986, 155, 77-88; Mason, P. W. et al. Virology 1987, 161, 262-267; Mackow, **E**. et al. Virology 1987, 159, 217-228; Sumiyoshi, H. et al. Virology 1987, 161, 497-510; Irie, K. et al. Gene 1989,. . .

SUMM . . . a vaccine which would protect against all four serotypes. However, despite more than 50 years of effort, safe and effective **dengue** virus vaccines have not been developed. Candidate vaccines currently being tested fall into two categories: live attenuated **dengue** virus vaccines and subunit vaccines, each with its own drawbacks.

SUMM . . . over-attenuated (fail to immunize). Even an optimally-attenuated live virus vaccine can revert to a virulent (disease-causing) form through mutation. Live **dengue** viruses are also sensitive to heat, making it difficult and costly to maintain the vaccine in some tropical and subtropical. . .

SUMM . . . subunit vaccines have the advantage of eliminating the risk of infectivity and greater chemical stability. However, the subunit vaccines of **flavivirus** structural and NS proteins produced in expression vectors including baculovirus, vaccinia virus and **E**. coli reported so far elicit only low titers of neutralizing antibody and are difficult to produce in large quantities and. . . al. Virology 1988, 163, 93-103; Putnak, J. R. et al. Am. J. Trop. Med. Hyg. 1991, 45, 159-167; Zhang, Y. **M**. et al. J. Virol. 1988, 62, 3027-3031; Lai, C. J. et al. In: Vaccines, Modern Approaches to New Vaccines Including. . . of AIDS (Eds. Lerner, R. A. et al.), Cold Spring Harbor Laboratory Press, New York, 89, 1989, pp. 351-356; Bray, **M**. et al. J. Virol. 1989, 63, 2853-2856; Bray, **M**. and Lai, C. J. Virology 1991, 185, 505-508; Men, R. et al. J. Virol. 1991, 65, 1400-1407; Mason, P. W.. . . al. J. Gen. Virol. 1989, 70, 2037-2049; Mason, P. W. et al. J. Gen. Virol 1990, 71, 2107-2114; Murray, J. **M**. et al. J. Gen. Virol, 1993, 74, 175-182; Preugschat, F. et al. J. Virol 1990, 64, 4364-4374).

SUMM Both the **envelope** (**E**) and the nonstructural protein 1 (NS1) are candidates for recombinant, subunit vaccines against DEN virus. The **E** glycoprotein is the major surface protein of the virion. It functions in virion attachment to host cells and it can be detected by its ability to hemagglutinate goose erythrocytes. As an antigen, it contains virus-neutralizing epitopes (Stevens, T. **M**. et al. Virology 1965, 27, 103-112; Smith, T. J. et al. J. Virol 1970, 5, 524-532; Rice, C. **M**. and Strauss, J. H. J. Mol. Biol. 1982, 154, 325-348; Brinton, **M**. A. In: Togaviridae and Flaviridae. Schlesinger, S. and **M**. J. Schlesinger

(Eds.), M. G. Plenum, New York, 1986, pp. 327-369; Heinz, F. X. Adv. Virus Res. 1986, 31, 103-168; Westaway, **E**. G. Adv. Virus Res. 1987, 33, 45-90; Hahn, Y. S. et al. Arch. Virol. 1990, 115, 251-265). Neutralizing antibodies, believed to correlate with protection, and hemagglutination-inhibiting (HI) antibodies develop following natural infection. Mice immunized with purified DEN-2 **E** antigen develop neutralizing antibodies and are protected against lethal virus challenge (Feighny, R. J. et al. Am. J. Trop. Med.. . .

SUMM . . . of developing a vaccine. Results have been variable and sometimes disappointing. Several strategies have been used to produce the DEN **E** protein in the baculovirus system. One strategy used a truncated gene to produce the **E** protein without the hydrophobic transmembrane segment of the carboxy terminus. The purpose of this approach was to promote secretion and. . . manner were minimally immunogenic in mice (Putnak, R. et al. Am. J. Trop. Med. Hyg., 1993, 45: 159-167; Zhang, Y. **M**. et al., J. Virol., 1988, 62: 3027-3031). Another strategy used a polygene that encoded the capsid, **premembrane** and two nonstructural proteins, C-**prM**-**E**-NS1-NS2 (Delenda et al. J. Gen. Virol, 1994, 75: 1569-1578). This construct produced the full length **E** protein by cleavage of the polyprotein. Neutralizing antibody to the full length **E** protein was not elicited by that product although protection was induced. The complex nature of the construct precludes an analysis. . . have induced the protective response. Another strategy employed a construct that contained a polygene encoding C, preM and a truncated **E** protein (Deubel et al. Virology, 1991, 180: 442-447). Although the truncated **E** reacted with some **E**-specific monoclonal antibodies (mAbs), reactivity was weaker than that obtained with native virus.

SUMM . . . a subunit vaccine that satisfies this need. The recombinant DEN virus subunit vaccine of the present invention comprises the full **dengue** virus **envelope** protein, expressed in baculovirus and capable of self-assembling into a particle. **Dengue envelope** protein has been expressed in the baculovirus system by others. The previously produced products were poorly immunogenic when tested in. . . previously made products are known to form particles. The protein is expressed and purified as a particle composed of multiple **dengue envelope** protein molecules. Particles are more immunogenic than soluble proteins, possibly because they can crosslink cell surface immunoglobulins on B cells. The **envelope** protein particle of the present invention is produced in baculovirus in large quantities and in pure form, elicits high titers. . .

SUMM The present invention describes the production of the DEN **envelope** protein particle by cloning the complementary DNA (cDNA) sequences encoding the **envelope** protein fragment into an expression vector such that the recombinant **dengue** protein can be expressed. The recombinant protein is produced in baculovirus, isolated and purified as a particle which is antigenic, reactive with **dengue** virus-specific and monoclonal antibodies and capable of eliciting the production of neutralizing antibodies when inoculated into mice. The administration of. . . recombinant subunit vaccine is demonstrated to protect mice, an accepted animal model, against morbidity and mortality following challenge with live **dengue** virus.

SUMM Therefore, it is an object of the present invention to provide a DEN 2 cDNA fragment encoding the full **envelope** glycoprotein, said gene containing 1485 nucleotides plus 93 adjacent upstream sequences and extending from 844 to 2422 of the viral. . .

SUMM It is another object of the invention to provide a recombinant vector designed to produce the recombinant DEN **envelope** protein for use as a vaccine and as a diagnostic agent.

SUMM It is still another object of the invention to provide a purified DEN **envelope** protein particle useful as a vaccine against DEN disease and for detecting the presence of said disease in a suspected. . .

SUMM It is another object of the present invention to provide a method for the purification of recombinant DEN **envelope** protein particle for use as a vaccine or as a diagnostic tool.

SUMM . . . DEN virus vaccine effective for the production of antigenic and

immunogenic response resulting in the protection of an animal against **dengue** virus disease.

DRWD FIGS. 1. (A-C) Illustration of the pBlueBacIII shuttle vector and gene sequences used for expression of the **dengue** 2 virus **envelope** glycoprotein in insect cells. A) illustration of relative positions of **dengue** 2 virus structural protein genes capsid ©, **premembrane** (**prM**) and **envelope** (**E**), and the N-terminal end of the adjacent non-structural protein NS1; B) nucleotide coordinates of the **E** gene construct used for insertion into shuttle vector pBluBacIII, extending from nucleotides 844 to 2422, including a sequence from vector. . .

DRWD FIG. 2. Gel filtration of **dengue** 2 virus recombinant **envelope** glycoprotein (rEgp) expressed by baculovirus using a column of G100 Sephadex. The column was equilbrated in phosphate buffered saline (PBS). . . PBS. Fractions were assayed for antigenic reactivity using the antigen dot blot assay and hyperimmune murine ascites fluid specific for **dengue** 2 virus. Data are plotted as absorbance at 280 nanometers (A260 nm) and counts per minute veses fraction number. Solid. . .

DRWD FIGS. 3. (A-B) Chromatographic analysis of recombinant **dengue** 2 virus **envelope** glycoprotein (rEgp) expressed by baculovirus using fast pressure liquid chromatography (FPLC) and a Superose 6 column. The column was equilibrated. . . with phosphate buffered saline (PBS) and protein was eluted with the same. A. Column fractions were assayed for antigen using anti-**dengue** 2 hyperimmune ascited fluid in a dot blot assay. Data are plotted as absorbance at 280 nanometers (A260 nm) and. . .

DRWD FIGS. 4. (A-D) Effect of sarkosyl on chromatographic elution profile of recombinant **dengue** 2 virus **envelope** glycoprotein (rEgp) analyzed using a Superose 6 column and fast pressure liquid chromatography (FPLC). The column was equilibrated in phosphate buffered saline (PBS) containing 0.1% sodium sarkosyl and protein containing recombinant **dengue** 2 **envelope** glycoprotein was eluted in PBS containing: A) 0.1% sarkosyl, B) 1.0% sarkosyl, C) 20% sarkosyl and D) 3.0% sarkosyl. Column fractions were assayed for antigenic activity using anti-**dengue** 2 hyperimmune ascites fluid in a dot blot assay. Data are plotted as absorbance (solid line) at 280 nanometers (A260). . .

DRWD FIGS. 5. (A-C) Effect of sonication on chromatographic elution profile of recombinant **dengue** 2 virus **envelope** glycoprotein (rEgp) analyzed usisng a Superose 6 column and fast pressure liquid chromatography (FPLC). Insect cells (Trichoplusia ni) infected with recombinant baculovirus expressing the **dengue** 2 virus **envelope** glycoprotein were sonicated in phosphate buffered saline (PBS) for 0, 20 and 30 minutes and eluted from a Superose 6. . . detected by absorbancy at 280 nanometers (A260) and dotted line (counts per minute) represents antigenic reactivity of fraction aliquots with anti-**dengue** 2 hyperimmune ascites fluid in a dot blot assay.

DRWD FIG. 6. Sucrose gradient centrifugation distribution of recombinant **dengue** 2 virus **envelope** glycoprotein (rEgp). Insect cells (Spodoptera frugiperda) infected with recombinant baculovirus were pelleted at low speed and protein remaining in the. . . a stp gradient of 5-30% sucrose in phosphate buffered saline (PBS). Fractions were assayed for antigenic activity (shaded area) using anti-**dengue** 2 hyperimmune ascites fluid in a dot blot assay.

DRWD FIGS. 7. (A-B) Polyacrylamide gelelectrophoresis and immunoblot analysis of baculovirus-expressed **dengue** 2 virus recombinant **envelope** glycoprotein (rEgp). The micorsomal pellet (described, FIG. 6) was ultracentrifuged through a cushion of 30% sucrose in phosphate buffered saline. . . B) Proteins were electrophoretically transferred to nitrocellulose paper and this immunoblot was probed with hyperimmune mouse ascites fluid specific for **dengue** 2 virus. Lanes in B correspond to lanes in A.

DETD In one embodiment, the present invention relates to a DNA or cDNA segment which encodes the complete **E** protein of DEN-2 and the carboxy terminus of **membrane/premembrane** protein extending from nucleotide 844 to 2422 of the DEN-2 viral genome and including linear and conformational, neutralizing epitopes said. . .

DETD . . . such sequences include regions encoding neutralizing epitopes present on the nucleotide sequence encompassing amino acids 1 through 495 of the **E** protein several of which have been mapped (Henchel, **E**. et al. Am. J. Trop. Med. Hyg., 1985, 34: 162-167) and found to be conformational as well as linear epitopes. . .

DETD . . . recombinant protein having an amino acid sequence corresponding to SEQ ID NO: 2 and encompassing 495 amino acids of the **E** protein and 31 amino acids of the carboxy-terminus of the adjacent **M/prM** protein from DEN-2 or any allelic variation thereof which maintains the neutralizing antibody production characteristic of the recombinant protein. As. . . can have an amino acid sequence corresponding to an epitope such as a B-cell and T-cell epitope present on the **envelope** glycoprotein of DEN-2, or conformational epitopes examples of which are found in TABLE 1. In addition, the protein or polypeptide,. . .

DETD . . . protein can be further stabilized by cross-linking reagents, and liposomes. The particle can encompass from at least 2 units of **envelope** protein. Such a particle can provide higher immunogenicity and possibly cross-link cell surface immunoglobulins on B cells.

DETD . . . methodology well known in the art. The recombinant protein can be used as a vaccine for immunity against infection with **flaviviruses** or as a diagnostic tool for detection of viral infection.

DETD (i) harvesting cells expressing recombinant DEN **envelope** glycoprotein;

DETD (iii) lysing said cell pellet of step (ii) to release recombinant **envelope** glycoprotein;

DETD (iv) pelleting said recombinant **envelope** glycoprotein from said lysed cells;

DETD (v) fractionating said recombinant **envelope** glycoprotein from steps (ii) and (v) through a density gradient;

DETD (vi) collecting purified recombinant **envelope** glycoprotein from pellet.

DETD . . . (vi) may be made of any density separation material such as cesium chloride, ficoll, or molecular sieve material. The recombinant **envelope** glycoprotein can also be pelleted from said supernatant. If desired, the cell debris can be pelleted or separated from said recombinant **envelope** glycoprotein after lysing cell pellet as described in (iii).

DETD . . . sample. Using standard methodology well known in the art, a diagnostic assay can be constructed by coating on a surface (i.**e**. a solid support) for example, a microtitration plate or a **membrane** (**e**.g. nitrocellulose **membrane**), all or a unique portion of the recombinant **envelope** protein particle described above, and contacting it with the serum of a person suspected of having DEN fever. The presence. . .

DETD In addition, the present invention is related to a method of detecting **flavivirus** disease or antibodies against **flavivirus** in a sample. **Dengue** viruses are members of the family Flaviridae which includes over sixty members among which there is considerable genetic and antigenic. . . apply the concepts of the present invention exemplified in DEN-2 to similar proteins and DNA sequences present in other related **flaviviruses** such as yellow fever, Japanese encephalitis and tick-borne **encephalitis viruses**.

DETD In another embodiment, the present invention relates to a diagnostic kit which contains the recombinant **envelope** protein particle and ancillary reagents that are well known in the art and that are suitable for use in detecting the presence of antibodies to **flavivirus** antigens in serum or a tissue sample, specifically antibodies to DEN virus. Tissue samples contemplated can be monkey and human,. . .

DETD In another embodiment, the present invention relates to a vaccine for protection against a **flavivirus** disease. The vaccine can be prepared by inducing expression of the recombinant expression vector described above in either a higher. . . can include preparing the particle under sterile conditions and adding an adjuvant. The vaccine can be lyophilized to produce a **flavivirus** vaccine in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form. . . and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the **dengue** vaccine and the side effects and adverse reactions are not increased

additively or synergistically. It is envisioned that a tetravalent vaccine composed of recombinant antigenic proteins from the four serotypes of **dengue** virus, DEN-1, DEN-2, DEN-3, and DEN-4 can be produced to provide protection against **dengue** disease.

DETD . . . ordinary skill in the art that due to the similarity between different serotypes of DEN as well as similarities between **flaviviruses**, a DNA sequence from any DEN serotype or **flavivirus** encoding the complete **envelope** protein of its respective **flavivirus** can be used as a naked DNA vaccine against infection with its respective virus. The DEN-2 naked DNA or RNA. . . or synergistically. It is envisioned that a tetravalent vaccine composed of DNA or RNA fragments from the four serotypes of **dengue** virus, DEN-1, DEN-2, DEN-3, and DEN-4 can be produced to provide protection against **dengue** disease.

DETD . . . naked DNA or RNA, as DNA or RNA encapsulated in liposomes, as DNA or RNA entrapped in proteoliposomes containing viral **envelope** receptor proteins (Nicolau, C. et al. Proc. Natl. Acad. Sci. U.S.A. 1983, 80, 1068; Kanoda, Y., et al. Science 1989,. . .

DETD Cells and viruses. **Dengue**-2 virus was propagated in Aedes albopictus cells (C6/36 cells, American Type Tissue Culture Collection, ATCC, Rockville, Md.). To propagate virus,. . .

DETD Cloning of the DEN-2 **envelope** gene. The gene encoding the DEN-2 Egp and an adjacent upstream translocation **signal sequence** (Markoff, L., J. Virol., 1989, 63:3345-3352) was derived by reverse transcription of viral genomic RNA followed by amplification of cDNA by the polymerase chain reaction. **Dengue**-2 virus RNA was purified from supernatants of virus-infected C6/36 cells by guanidine isothiocyanatephenol chloroform:isoamyl alcohol extraction (Chomczynski and Sacchi, Anal. . .

DETD . . . incubated for 1 hour in blocking buffer containing goat anti-mouse immunoglobulin gamma (Kirkegaard and Perry) labeled with ^{125}I (Gentry **M**. K. et al., Am. J. Trop. Med. Hyg., 1982, 31: 548-555), using labeled antibody at 10^6 cpm/ml of blocking buffer.. . .

DETD . . . fragment encodes the full Egp (495 amino acids) and 31 amino acids of the C terminus of the adjacent upstream **M**/preM protein. This segment serves as a signal for **membrane** translocation of the Egp (Markoff, L. J. Virol. 1989, 63:3345-3352)). Synthetic primers used to amplify the gene fragment each contained 18 nucleotides complementary to specific sequences in the DEN-2 **E** gene. The forward primer contains a Bgl II enzyme restriction site and an ATG start codon (SEQ ID NO:3). The reverse primer contains a Pst I enzyme restriction site and a stop codon. The **E** gene fragment was cut with Bgl II and Pst I enzymes and inserted unidirectionally into the BglII-Pst I cloning site. . .

DETD TABLE 1

Antibody binding of the **dengue**-2 recombinant **envelope** protein expressed by baculovirus

Antibody[a]	Reactivity with antigen[b]		
	ACNPV-**E**	ACNPV-prME	DEN-2 Virus ACNPV
3H5[d]	13.6[c]	10.1	7.5 1.4
9D12[d], **e**	12.3	12.1	9.0 1.0
13B7	10.5	4.1	5.6 3.6
4E5[d]	8.6	6.6	10.5 1.0
2H3[d]	4.9	2.5	11.5 1.9
4G2[d], **e**	8.5	5.0	16.8 1.0
1B7[d], **e**	5.1	3.1	8.9 1.2
HMAF	13.5	17.6	7.0 1.2
HCS	12.5	NT	12.5 1.6

[a] Antibodies were diluted 1:100 (mAbs). . . × 10^3.

[d] Antibodies which neutralize virus infectivity in vitro (Henchal et al. Am J. Trop. Med. Hyg. 1985, 34:162-167).
[e] Antibodies which recognize conformational epitopes (Henchal et al. Am J. Trop. Med. Hyg. 1985, 34:162-167; Megret et al. Virology, 1992,
. . .
DETD TABLE 2

Binding affinity of monoclonal antibodies to recombinant and native **dengue**-2 **envelope** proteins

Affinity binding of mAbs 9D12, 2H3, and 4G2 at pH 5.0			
Antigen[a]	9D12	2H3	4G2
Purified	0.4×10^{-6}	3.2. . . 10^{-6}	2.9×10^{-6}
Virus	5.2×10^{-6}	2.0×10^{-6}	1.3×10^{-6}

[a] Antigens were either partiallypurified recombinant **E** protein, lysates of cells infected with the Eprotein recombinant baculovirus, or purified DEN2 virus.

DETD The mAb binding assays qualitatively demonstrate that native protein epitopes were preserved on the recombinant **E** protein.

DETD Purification of **E** particles. Gel filtration results indicated that rEgp aggregates could be separated from the majority of cellular proteins based on their. . . pellet, compared to relatively small amount of antigenic activity that was distributed into several gradient fractions. Since the majority of **E** antigen was present in the 5-30% sucrose gradient pellet, **E** protein aggregates were purified by centrifugation of the microsomal fraction through a 30% sucrose cushion.

DETD . . . trials in mice. Previously it was shown that a cellular lysate containing baculovirus vectored rEgp was fully reactive with native **E**-specific monoclonal antibodies and induced a low titer of neutralizing antibody in mice. Table 3 shows results from immunization of mice. . .

DETD TABLE 3

$PRNT_{50}$ in mice immunized with baculovirus expressed DEN-2 **E** protein

rEgp with Alum	rEgp/no adjuvant
Dose 4 µg	
Dose 1 µg	
Dose 4 µg	
Dose 1 µg	
DEN-2 NGC	
Control	

>850. . .

DETD TABLE 4

Percent protection measured by reduction of **dengue** virus in the brains of immunized, intracerebrally challenged mice

Immunization[1]	Percent reduction[2]
4 µg with alum	88.5 ± 35.1
1 µg. . .	± 0
none	0 ± 86

[1] Mice were immunized at days 0 and 30 with indicated amounts of recombinant **dengue** 2 **envelope** protein or with live **dengue** 2 virus. Contro mice were not immunized.

[2] Mice were inoculated intracerebrally with 10,000 pfu of mouse adapted **dengue** 2 virus two weeks after the last immunization. Five days

later, mice were euthanized and the brains removed for quantitation of **dengue** virus in the brain. The percent reduction was calculated by multiplying 100 times the formula (controlplagues/control) where control is the. . .

DETD . . . - # 120 - # 125

- - Asp Arg Gly Trp Gly Asn Gly Cys Gly Leu Ph - #e Gly Lys Gly Gly Ile
130 - # 135 - # 140

- - Val Thr Cys Ala Met Phe. . . #90 3 - #95
4 -

#00

- - Glu Pro Pro Phe Gly Gln Ser Tyr Ile Ile Il - #e Gly Val Glu Pro Gly
405 - # 410 - # 415

- - Gln Leu Lys Leu Asp Trp Phe. . .

1. An isolated and purified **dengue** virus DNA fragment consisting essentially of a DNA fragment which encodes a complete **dengue** virus **envelope** protein and a carboxy terminus segment of **premembrane** protein which comprises a translocation signal for said **Envelope** protein.

2. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** virus is **dengue** 2.

3. The DNA fragment of claim 2 which encodes 495 amino acids of said **envelope** protein and 31 amino acids of said carboxy terminus segment of **premembrane** protein, said fragment comprising the nucleotide sequence specified in SEQ ID NO: 1 or an allelic variant which retains the. . .

5. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** is **dengue** 1.

6. The isolated and purified DNA fragment according to claim 1, wherein said **dengue** is selected from the group consisting of **dengue** 3 and **dengue** 4.

7. A recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

8. A recombinant DNA construct according to claim 7, wherein said **dengue** virus is **dengue** 2.

11. A recombinant DNA construct comprising: (i) a vector, and (ii) a **dengue** 2 DNA fragment according to claim 3.

. . . 15. A host cell transformed with a recombinant DNA construct comprising: (i) a vector, and (ii) an isolated and purified **dengue** virus DNA fragment according to claim 1.

18. A method for producing a **dengue** virus recombinant protein particle, said method comprising the steps of: (i) culturing a host cell transformed with an expression vector. . .

19. The method according to claim 18, wherein said **dengue** virus is **dengue** 2.

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2000:9526 cDNA sequence of **Dengue** virus serotype 1 (Singapore strain).

Fu, Jianlin, Singapore, Singapore
Tan, Boon-Huan, Singapore, Singapore
Yap, Eu-Hian, Singapore, Singapore
Chan, Yow-Cheong, Singapore, Singapore
Tan, Yin-Hwee, Singapore, Singapore
Insititute of Molecular and Cell Biology, Singapore (non-U.S. corporation)

US 6017535 20000125
WO 9322440 19931111
APPLICATION: US 1994-325426 19941216 (8) <--
WO 1993-CA182 19930428 19941216 PCT 371 date 19941216 PCT 102(e) date

PRIORITY: GB 1992 9245 19920429
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB DENI-S275/90 (ECACC V92042111) is a new strain of **Dengue** virus serotype 1. The complete cDNA sequence of this virus has been cloned and protein-coding fragments thereof have been used in the construction of expression plasmids. DEN1-S275/90 in inactivated form, DEN1-S275/90 polypeptides or fusion proteins thereof can be incorporated into vaccines for immunisation against DEN1-S275/90 and other DEN1 viruses. The invention further provides diagnostic reagents **e**.g. labelled antibodies to DEN1-S275/90 proteins, and kits to detect DEN1 virus.

CLM What is claimed is:

1. An isolated **Dengue** viral strain DEN1-S275/90 designated as (**E**-CACC V92042111).

2. An isolated **Dengue** viral strain DEN1-S275/90 designated as (ECACC V92042111), in inactivated form.

3. An isolated DNA polynucleotide encoding DEN-1-S275/90 (ECACC V92042111) consisting of the sequence shown in SEQ ID NO:1 or at least one nucleotide sequence selected from the group consisting of nucleic acids 81-422, 123-422, 423-695, 696-920, 921-2402, 2403-3464, 3465-4112, 4113-4499, 4500-6359, 6360-6809, 6810-7556, and 7557-10268, of SEQ ID NO:1.

4. An isolated DNA polynucleotide encoding the polypeptide of SEQ ID NO:2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2.

5. An isolated DNA polynucleotide encoding a fusion protein comprising the polypeptide of SEQ ID NO: 2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2.

6. An expression vector comprising the DNA polynucleotide of any one of claims 3-4.

7. The expression vector according to claim 6, said vector being a plasmid or viral vector.

8. An expression vector comprising the isolated DNA polynucleotide of claim 5.

9. A cell containing the expression vector according to claim 6.

10. The cell according to claim 9, said cell being **E**. coli, a yeast cell or an insect cell.

11. A cell containing the expression vector of claim 8.

12. A method of preparing a polypeptide having an amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396, of SEQ ID NO:2, said method comprising culturing a cell according to claim 9 and recovering said polypeptide.

13. A method of preparing a fusion protein comprising the polypeptide of SEQ ID NO: 2 or at least one amino acid sequence selected from the group consisting of amino acids 1-114, 15-114, 115-205, 206-280, 281-774, 775-1128,1129-1344, 1345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396 of SEQ ID NO:2, said method comprising culturing a cell according to claim 11 and recovering said fusion protein.

14. A polypeptide in isolated form which is selected from the group consisting of amino acids 1-14, 15-114, 115-205, 206-280, 281-774, 775-1128, 1129-1344, 345-1474, 1475-2093, 2094-2242, 2243-2492 and 2493-3396, of SEQ ID NO:2.

15. An isolated fusion protein comprising the polypeptide according to claim 14.

16. A polypeptide according to claim 14, attached to a label.

17. A fusion protein according to claim 15 attached to a label.

18. A test kit for the detection of the presence or absence of DEN1 virus antibodies comprising a polypeptide according to any one of claims 14-17 fixed to a solid support, said polypeptide being capable of binding antibodies to DEN1-S275/90 (ECACC V92042111).

19. A method of preparing antibodies in an animal, said antibodies being capable of binding a **Dengue** virus viral protein, said method comprising immunizing said animal with at least one polypeptide according to any one of claims 14-17 or the inactivated virus according to claim 2 and isolating said antibodies.

20. A method as claimed in claim 19 which further comprises in vitro labeling one or more isolated antibodies capable of binding a **Dengue** viral protein.

21. An isolated antibody prepared according to the method of claim 19.

TI cDNA sequence of **Dengue** virus serotype 1 (Singapore strain)

AI US 1994-325426 19941216 (8) <--
WO 1993-CA182 19930428
19941216 PCT 371 date
19941216 PCT 102(e) date

AB DENI-S275/90 (ECACC V92042111) is a new strain of **Dengue** virus serotype 1. The complete cDNA sequence of this virus has been cloned and protein-coding fragments thereof have been used. . . thereof can be incorporated into vaccines for immunisation against DEN1-S275/90 and other DEN1 viruses. The invention further provides diagnostic reagents **e**.g. labelled antibodies to DEN1-S275/90 proteins, and kits to detect DEN1 virus.

SUMM The present invention relates to **Dengue** Virus Type 1. **Dengue** virus infection may lead to **dengue** fever (DF) or its more severe **dengue** haemorrhagic fever (DHF) and **dengue** shock syndrome (DSS). DHF is an important virus disease of global significance, especially in Southeast Asia. There are four serotypes of **Dengue** virus (DEN1, DEN2, DEN3 and DEN4) belonging to the family **Flaviviradae**.

SUMM . . . provides the nucleic acid sequence of Seq. ID No. 1 and DNA sequences substantially corresponding to SEQ ID No. 1, **e**.g. degenerate variants thereof having one or more nucleotide changes but nevertheless capable of being translated to give the same protein sequence. The invention further provides fragments of such DNA polynucleotides, in particular the fragments encoding the C, C', PreM, **M**, **E**, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 genes of the genome of the virus. The start and end points. . .

SUMM . . . S275/90.

Gene	Start(n)	End(n)	Start(p)	End(p)
C	81	422	1	114
C'	123	422	15	114
PreM	423	695	115	205
M	696	920	206	280
E	921	2402	281	774

NS1 2405 3464 775 1128
NS2A 3465 4112 1129 1344
NS2B 4113 4499 1345 1474
NS3. . .

SUMM . . . set forth in Seq. ID No. 1 and Seq. ID No. 2 and fragments thereof, eg. the C, C', PreM, **M**, **E**, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 proteins as identified above in Table 1. The invention thus provides a. . .

SUMM . . . may be selected to be suitable to express the nucleic acid sequences of the invention in, for example, a bacterial **e**.g. **E**. coli, yeast, insect or mammalian cell. A baculovirus expression system may be used. The nucleic acid may be expressed in. . . is produced or alternatively it may be expressed to provide a fusion protein in which DEN1-S275/90 or a protein thereof, **e**.g. **E**, NS1, NS2, NS3 or NS5 as identified in Table 1 above is fused to a second amino acid sequence, **e**.g. a C-terminal sequence derived from glutathione S-transferase or maltose binding protein or a C-terminal or N-terminal **signal sequence**. Such a sequence may for example cause the fusion protein to be exported from the cell. The expression vector is. . .

SUMM . . . more peptides. For example, it may comprise one non-structural (NS) peptide, eg. NS1 or NS3, together with a capsid (C), **M** or **E** peptide. A mixture of two or more NS peptides could also be used.

SUMM . . . isotope selected to kill virus-infected cells. Antibodies against NS1 are particularly favoured since NS1 is expressed on the surface of **Dengue** virus-infected cells.

DRWD FIG. 1 is a diagrammatic representation of the cDNA of **Dengue** virus Type 1 (Singapore strain S275/90) and fragments of said DNA in expression vectors;

DRWD Gel Lanes: Lane 1: **M**, Lane 2: anti **E**, Lane 3: anti-**E**+ CFA, Lane 4: anti-NS1, Lane 5: anti-NS2, Lane 6: anti-NS2+ CFA, Lane 7: anti-NS3, Lane 8: anti-NS3+ CFA, Lane 9: anti-NS5, Lane 10: anti-NS5+ CFA, Lane 11: positive rabbit sera, Lane 12: negative rabbit sera, Lane 13: **M**;

DRWD Gel Lanes: Lane 1: (-), Lane 2: (+) anti-**E**, Lane 3: (-), Lane 4: (+) anti-NS1, Lane 5: (-), Lane 6: (+) anti-NS2, Lane 7: (-), Lane 8: (+) anti-NS3, Lane 9: (-), Lane 10: (+) anti-NS5, Lane 11: positive **Dengue**, Lane 12: patient sera;

DRWD FIG. 4 shows fluorescence microscopy of C6/36 cells infected with **Dengue** Type 1 DI-275 and probed with antibodies against recombinant fusion proteins. A, control antiserum; B, anti-**E**; C, anti-NS1; D, anti-NS2; **E**, anti-NS3; F anti-NS5.

DETD . . . the assorted cDNAs were cloned into EcoRI sites of pUC18 vector via EcoRI adaptors (Promega). The Esherichia coli transformants containing **Dengue**-specific sequences were screened by colony hybridisation with ^{32} P-labelled cDNA probes prepared by reverse transcription of strain S275/90 RNA. The. . .

DETD Potential secondary structures have been postulated for the 5' and 3' ends of **flaviviruses** (4, 7, 8), posing a problem in obtaining clones with intact ends. A different stragegy for sequencing the 5' and. . . the other end. For the 3' noncoding region, an additional step was included before cDNA synthesis. After extraction, the purified **Dengue** viral RNA was tailed by poly A polymerase (Bethesda Research Laboratories) with ATP. This was followed by cDNA synthesis using. . .

DETD . . . sequence data obtained from the overlapping cDNA clones was ordered by homology alignment with the published sequences of the four **Dengue** serotypes DEN1, DEN2, DEN3 and DEN4 using the computer program of Wilbur and Lipman (9). Seq ID No. 1 shows. . . the 5' noncoding region preceding the first AUG codon of the open reading frame appears to be conserved for all **Dengue** virus types (1-4). The length of the 3' noncoding region of strain S275/90 is longer than that of DEN2 (412. . .

DETD . . . nucleotide composition of strain S275/90 is 31.9% A, 25.9% G, 21.5% T and 20.7% C. As reported for the other **flaviviruses**, the same purine-rich composition was observed, and there is an absence of poly(A) tract at the 3' end.

DETD . . . individual protein coding segments are based on comparison with protein sequence data for all the proteins determined from the four

Dengue serotypes. These cleavage sites may reveal the involvement of viral or cellular proteases involved in protein processing. The C, preM, **M**, **E**, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 proteins are cleaved at the sites **M**/MNQRKK, A/FXL, RXKR/SV, X/MRCXG, VQA/DXGCV, VXA/GXG, X/SWPLN, KXQR/XG, GRX/S, VXA/NE (SEQ ID NO:s 16-25, respectively) and R/G, respectively, where X refers. . .

DETD . . . nucleotide and amino acid changes, and hence the greatest evolution, lie in the nonstructural gene NS2A in all the four **Dengue** serotypes. A high homology is found in NS3 and NS5, which contain conserved sequences.

DETD Chu et al (11) compared three topotypes of DEN1 strains (Thailand, Philippines and Caribbean) genetically at the **envelope** region. They found nucleotide changes to be less than 5% but translational differences of 2% at the amino acid level. Our strain S275/90 shows nucleotide changes of 7.7% and amino acid changes of 2.6% in the **envelope** region. Rico-Hesse (6) compared nucleotide sequences within a chosen **E**/NS1 region to estimate evolutionary relationships among 40 DEN1 strains of different geographic range and time period.

DETD TABLE 2

HOMOLOGY (%) COMPARISON OF ALIGNED NUCLEOTIDE SEQUENCES OF THE FOUR **DENGUE** SEROTYPES WITH STRAIN S275/90 (AMINO ACID ALIGNMENT WITHIN BRACKETS).

S275/90	DEN1	DEN2	DEN3	DEN4
Full	93.1 (97.6)	67.1 (70.9)	70.4 (75.5)	65.1 (67.6)
length				
5' non-coding	100	81.7	93.8	87.7
C	97.4 (98.2)	70.5 (67.5)	80.5 (80.7)	68.1 (67.9)
PrM	91.6 (95.6)	71.1 (75.8)	75.8 (78.0)	68.0 (68.1)
M	93.3 (98.7)	64.0 (70.7)	70.3 (78.7)	60.7 (60.3)
E	92.3 (97.4)	65.4 (67.7)	69.0 (76.4)	64.8 (61.8)
NS1	92.6 (98.0)	70.1 (73.6)	74.5 (78.7)	70.1 (68.8)
NS2A	--	55.1. . .		

DETD 1. WESTAWAY, **E**. G., BRINTON, **M**. A., GAIDAMOVICH, S. Ya, HORZINEK, **M**. C., IGARASHI, A., KAARIAINEN, L., LVOV, D. K., PORTERFIELD, J. S., RUSSEL, P. K., and TRENT, D. W., Intervirology 24,. . .

DETD 4. DEUBEL, V., KINNEY, R. **M**., and TRENT, D. W., Virology 165, 234-244 (1988).

DETD 6. RICO-HESSE, R., PALLANSCH, **M**. A., NOTTAY, B. K., AND KEW, O. **M**., Virology 174, 479-493 (1990)

DETD 7. BRINTON, **M**. A., and DISPOTO, J. H., Virology 162, 290-299 (1988).

DETD 8. IRIE, K., MOHAN, P. **M**., SASAGURI, Y., PUTNAK, R. and PADMANABHAN, R., Gene 75, 197-211 (1989).

DETD 10. RICE, C. **M**., LENCHES, **E**. **M**., EDDY, S. R., SHIN, S. J., SHEETS, R. L., and STRAUSS, J. H., Science 229, 726-733 (1985).

DETD 11. CHU, **M**. C., O'ROURKE, **E**. J. and TRENT, D. W., J. Gen. Virol. 70, 1701-1712 (1989).

DETD For construction of plasmids, the cDNA regions for **E**, NS1, NS2, NS3 and NS5 of clone DI-275, a DEN1 cDNA clone derived from DEN1 virus Singapore Strain S275/90 as. . .

DETD Fragments of **E**, NS3 and NS5 cDNA digested with restriction enzymes were ligated to the pGEX-KG vector (Guan and Dixon, Anal. Biochem. 192,. . . digestion. The two parts of NS5 were ligated together, then ligated into the pGEX-KG vector. Recombinant plasmids were transformed into **E**. coli DH5α or c600 HF1 strains. All plasmids encoded **Dengue** virus proteins fused to the C-terminus of glutathione S-transferase or Maltose Binding Protein (MBP).

DETD Purification of **E**, Ns3 And Ns5 Proteins from Recombinant **E**. Coli

DETD **E**. coli, harbouring **E**, NS3 and NS5 genes (separately) were grown in LB medium A_{600} of 0.5 at 37° C., then induced with IPTG. . . 0.2 mM for 2 h at 30° C. The bacteria were harvested and

resuspended on ice in MTBS buffer (0.15 **M** NaCl, 0.010 **M** Na_2HPO_4, 0.005 **M** NaH_2PO_4) with 0.1 mg/ml lysozyme, 1% triton X-100, 0.5 μg/ml aprotinin, 0.05 μg/ml Leupeptin, 0.25 μg/ml pepstatin, 5 mM. . . the wash solution read zero at A_{280} in a spectrophotometer. The beads were resuspended in thrombin cleavage buffer, and the **Dengue** virus proteins were cleaved off the beads with thrombin at 4° C. for 1 hr. The supernatant, containing **Dengue** virus proteins, was recovered by centrifugation, and the proteins were stored at -80° C.

DETD **E**. coli containing the NS1 fusion protein was grown as above, except the tac promoters were induced with 0.3 mM IPTG for 16 h. The bacteria were harvested, 1 gram wet weight of **E**. coli was resuspended in 5 ml lysis buffer with lysozyme at 1.6 mg/ml and was sonicated for 2×15 sec. After. . . mM EDTA, and 100 mM NaCl, then centrifuged at 20,000× g twice. The pellet was washed with 1 ml 2 **M** urea twice and dissolved in 8 **M** urea in 0.1 **M** Tris-HCl pH 8.8, 0.14 **M** 2-mercaptoethanol. The urea concentration was reduced to 1 **M** by adding H_2O, and amylose resin (New England Biolabs) was added to adsorb the solubilised fusion protein at 22°. . .

DETD After growth of **E**. Coli transformed with pMAL-cRI/NS2-1, lysis and sonication as in Example 3 above, the clarified extract containing the soluble NS2 fusion. . .

DETD The soluble fusion proteins of **E**, NS2, NS3 and NS5 purified from recombinant **E**. coli, as in Examples 3 and 5 above, and inclusion bodies containing the NS1 fusion protein which had been purified. . . the last booster dose. For immunisation of mice, 12-day old female Swiss mice were immunised with the soluble proteins of **E**, NS1, NS2, NS3 and NS5 fusion proteins with or without Freund's adjuvant. The injections were intraperitoneal or subcutaneous on the. . .

DETD Radioimmunoprecipitations were done with rabbit and mouse antibodies against the structural and non-structural **Dengue** virus recombinant fusion proteins of D-275. At 36-40 h post-infection of C6/36 cells with **Dengue** virus S275/90 strain, cell culture medium was replaced with methionine-free medium containing 3 μg/ml actinomycin D for 3 h, followed. . . protein A-Sepharose and were washed with immunoprecipitation buffer [10 mM Tris-HCl, pH7.4, 0.05% aprotinin, 1% NP40, 2 mM EDTA, 0.15 **M** NaCl], 6 times then 2× SDS-PAGE buffer was added, boiled for 2 min, and the supernatant was loaded on a. . . gel. After fixing enhancing and drying, the gel was exposed to X-ray film. The results confirmed that antibodies to recombinant **E**, NS1, NS2, NS3 and NS5 had been generated in mice (FIG. 2) and in rabbits (FIG. 3). These antibodies reacted with the native **E**, NS1, NS2, NS3 and NS5 proteins synthesised in infected C6/36 cells.

DETD The C6/36 cells infected with **Dengue** virus S275/90 for 2 days were fixed on glass plates with cold acetone for immunofluorescence. 2-fold dilutions of the sera. . . incubated for 1 h, followed by washing with PBS for observation using fluorescence microscopy. FIG. 4 shows the antisera to **E**, NS1, NS2, NS3 and NS5 reacted specifically with the **Dengue** virus S275/90 infected cells, but control antiserum did no react. Quantitation of the result (as set out in Table 4) showed that an immune response to all recombinant **Dengue** virus proteins (**E**, NS1, NS2, NS3 and NS5) occurred in both mice and rabbits.

DETD TABLE 3

Oligonucleotides used to prepare cDNA fragments corresponding to **Dengue** virus proteins (by PCR)

#STR1##

#STR2##

#STR3##

#STR4##

DETD TABLE 4

IMMUNE RESPONSES OF MICE AND RABBITS: INDIRECT IMMUNOFLUORESCENCE ASSAYS

Dengue virus type 1 recombinant proteins	No. of mice	Σ Titrations of IFA
E	11	14.91
E + CFA	10	39.62
NS1	10	14.89
NS2	10	12.05
NS2 + CFA	10	12.07
NS3	11	10.94
NS3 + CFA	10	42.56
NS5	10	7.94
NS5 + CFA	10	10.47
E + NS1	17	16.66
NS3 + NS1	18	10.87
NS2 + NS3	14	9.23
NS5 + NS3	10	32.14
MBP	4	<4
GST	4	<4
PBS	2	<4

Dengue virus type 1 recombinant proteins	Σ Titrations of rabbits IFA
E	1 160
NS1	1 160
NS2 (67)	1 2560
NS2 (68)	1 640
NS3	1 2560
NS5	1 160

DETD . . . RNA of DEN1-S275/90
- - (iii) HYPOTHETICAL: NO
- - (iv) ANTI-SENSE: NO
- - (vi) ORIGINAL SOURCE:
(A) ORGANISM: **Dengue** Fe - #ver Virus Type 1
(B) STRAIN: S275/90
- - (ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 81..10268
-. . . - #C CTA AGA TTT CTA GCC
254
Pro Met Lys Leu Val Met Ala Phe Ile Ala Ph - #e Leu Arg Phe Leu Ala
45 - # 50 - # 55
- - ATA CCC CCA ACA GCA GGA. . . AT - #T GAA GCT AAA ATA
TCA 1118
Thr Asn Pro Ala Val Leu Arg Lys Leu Cys Il - #e Glu Ala Lys Ile Ser
335 - # 340 - # 345
- - AAC ACC ACC ACC GAT TCA. . . - #A CAG CTG ACC GAC TAC
1454
Thr Ile Thr Pro Gln Ala Pro Thr Ser Glu Il - #e Gln Leu Thr Asp Tyr
445 - # 450 - # 455
- - GGA GCC CTC ACA TTG GAC. . . - #A GGG AAA ATG TTC GAA
2126
Lys Gln Cys Trp Phe Lys Lys Gly Ser Ser Il - #e Gly Lys Met Phe Glu
670 - # 675 - # 680
- - GCA ACC GCC CGA GGA GCA. . . - #C CTG GGA GAC ACC GCA
2174

```
Ala Thr Ala Arg Gly Ala Arg Arg Met Ala Il - #e Leu Gly Asp Thr Ala
        685          - #       690          - #       695
  - - TGG GAC TTC GGT TCT ATA.  .  .  - #C AGA CCA GTC AAG GAA
    3422
 Gly Glu Asp Gly Cys Trp Tyr Gly Met Glu Il - #e Arg Pro Val Lys Glu
     1100             - #   1105             - #  1110
  - - AAG GAA GAG AAT CTA GTC.  .  .  - #A TCA ATA ATG ATC GAA
    3518
 Val Asp Ser Phe Ser Leu Gly Leu Leu Cys Il - #e Ser Ile Met Ile Glu
                1135 - #               1140  - #              1145
  - - GAG GTG ATG AGA TCC AGA.  .  .  - #T TCC TTG CAC TAT GCA
    3950
 Leu Leu Ser Leu Thr Phe Val Lys Thr Thr Ph - #e Ser Leu His Tyr Ala
 1275               1280 - #               1285 - #               1290
  - - TGG AAG ACA ATG GCT.  .  .  - #T CTC ATA GCA GAA AAC
    4094
 Gly Ser Leu Gly Cys Lys Pro Leu Thr Met Ph - #e Leu Ile Ala Glu Asn
        1325         - #       1330         - #       1335
  - - AAA ATC TGG GGA AGG AAA.  .  .
DETD     .  .  .  - #T GAG GAC GAG GTG TTT
    5054
 Lys Ala Ser Gln Glu Gly Pro Leu Pro Glu Il - #e Glu Asp Glu Val Phe
        1645         - #       1650         - #       1655
  - - AGG AAA AGA AAC TTA ACA.  .  .  - #C CCA GGT AAA ACA GTC
    5582
 Trp Asn Ser Gly Tyr Glu Trp Ile Thr Asp Ph - #e Pro Gly Lys Thr Val
     1820             - #   1825             - #  1830
  - - TGG TTT GTT CCA AGC ATC.  .  .  - #A AAC ACA CCA GAA GGG
    6014
 Trp Thr Glu Ala Lys Met Leu Leu Asp Asn Il - #e Asn Thr Pro Glu Gly
        1965         - #       1970         - #       1975
  - - ATC ATC CCA GCC CTC TTT.  .  .  - #T AAA GAG TTT GCA GCA
    6350
 Thr Tyr Ser Asp Pro Leu Ala Leu Arg Glu Ph - #e Lys Glu Phe Ala Ala
 2075               2080 - #               2085 - #               2090
  - - GGA AGA AGA AGT GTC.  .  .  - #C CTA TCA GGA AAG GGC
    6590
 Ala Val Leu Thr Gly Gly Val Thr Leu Phe Ph - #e Leu Ser Gly Lys Gly
 2155               2160 - #               2165 - #               2170
  - - CTA GGG AAA ACA TCT.  .  .  - #T CCA GAG CCA GAC AGA
    6734
 Ile Leu Glu Phe Phe Leu Met Val Leu Leu Il - #e Pro Glu Pro Asp Arg
        2205         - #       2210         - #       2215
  - - CAG CGC ACT CCA CAG GAC.  .  .  - #C AGG GGA AGT TAT CTA
    7502
 Thr Thr Ile Ala Val Ser Met Ala Asn Ile Ph - #e Arg Gly Ser Tyr Leu
     2460             - #   2465             - #  2470
  - - GCA GGA GCA GGC CTG GCC.  .  .  - #C AAC ACT TAC AAA AGG
    7646
 Asp Arg Leu Asn Gln Leu Ser Lys Ser Glu Ph - #e Asn Thr Tyr Lys Arg
            2510     - #           2515     - #          2520
  - - AGT GGG ATT ATG GAA GTG.  .  .  - #A TGG TAC ATG TGG TTG
    8990
 Glu Phe Gly Lys Ala Lys Gly Ser Arg Ala Il - #e Trp Tyr Met Trp Leu
 2955               2960 - #               2965 - #               2970
  - - GGA GCA CGC TTT CTA.  .  .  - #A TCA AAG ATT CCA GGG
    9134
 Leu His Lys Leu Gly Tyr Ile Leu Arg Asp Il - #e Ser Lys Ile Pro Gly
        3005         - #       3010         - #       3015
  - - GGA AAT ATG TAT GCA GAT.  .  .
DETD     .  .  .  - #C GGG AAT GAG AAT TAT
   10190
 Gln Val Ala Ile Asn Gln Val Arg Arg Leu Il - #e Gly Asn Glu Asn Tyr
 3355               3360 - #               3365 - #               3370
  - - CTA GAT TAC ATG ACA.  .  .  - #          40          - #          45
  - - Ala Phe Ile Ala Phe Leu Arg Phe Leu Ala Il - #e Pro Pro Thr Ala Gly
```

- - Ile Leu Ala Arg Trp Gly. . . - # 120 - # 125
- - Ser Lys Gln Glu Arg Glu Lys Ser Leu Leu Ph - #e Lys Thr Ser Val Gly
130 - # 135 - # 140
- - Val Asn Met Cys Thr Leu. . . - # 250 - #
255
- - Ala Ile Gly Thr Ser Ile Thr Gln Lys Gly Il - #e Ile Phe Ile Leu Leu
260 - # 265 - # 270
- - Met Leu Val Thr Pro Ser. . . - # 890 - #
895
- - Ile Ile Gly Ala Asp Ile Gln Asn Thr Thr Ph - #e Ile Ile Asp Gly Pro
900 - # 905 - # 910
- - Asp Thr Pro Glu Cys Pro. . . - # 1015 - # 1020
- - Ile Ser Gln His Asn Tyr Arg Pro Gly Tyr Ph - #e Thr Gln Thr Ala Gly
1025 1030 - # 1035 - # 1040
- - Pro Trp His Leu Gly Lys Leu Glu Leu Asp Ph - #e Asp Leu Cys Glu Gly
1045 - # 1050 - # 1055
- - Thr Thr Val Val Val Asp. . . - # 1240 - # 1245
- - Leu Gly Asp Gly Leu Ala Met Gly Ile Met Il - #e Leu Lys Leu Leu Thr
1250 - # 1255 - # 1260
- - Asp Phe Gln Ser His Gln. . . - # 1450 - #
1455
- - Ser Ile Pro Ala Thr Leu Phe Val Trp Tyr Ph - #e Trp Gln Lys Lys Lys
1460 - # 1465 - # 1470
- - Gln Arg Ser Gly Val Leu. . . - # 1480 - # 1485
- - Arg Ala Val Leu Asp Asp Gly Ile Tyr Arg Il - #e Met Gln Arg Gly Leu
1490 - # 1495 - # 1500
- - Leu Gly Arg Ser Gln Val Gly Val Gly Val Ph - #e Gln Asp Gly Val Phe
1505 1510 - # 1515 - # 1520
- - His Thr Met Trp His. . . - # 1770 - # 1775
- - Thr Arg Val Gly Met Gly Glu Ala Ala Ala Il - #e Phe Met Thr Ala Thr
1780 - # 1785 - # 1790
- - Pro Pro Gly Ser Val Glu. . . - # 1960 - # 1965
- - Leu Leu Asp Asn Ile Asn Thr Pro Glu Gly Il - #e Ile Pro Ala Leu Phe
1970 - # 1975 - # 1980
- - Glu Pro Glu Arg Glu Lys. . .
DETD . . . - # 2185 - # 2190
- - Val Glu Pro His Trp Ile Ala Ala Ser Ile Il - #e Leu Glu Phe Phe Leu
2195 - # 2200 - # 2205
- - Met Val Leu Leu Ile Pro. . . - # 2855 - # 2860
- - Met Glu Val Thr Ala Arg Trp Leu Trp Gly Ph - #e Leu Ser Arg Asn Lys
2865 2870 - # 2875 - # 2880
- - Lys Pro Arg Ile Cys. . .

1. An isolated **Dengue** viral strain DEN1-S275/90 designated as (**E**-CACC V92042111).

2. An isolated **Dengue** viral strain DEN1-S275/90 designated as (ECACC V92042111), in inactivated form.

10. The cell according to claim 9, said cell being **E**. coli, a yeast cell or an insect cell.

19. A method of preparing antibodies in an animal, said antibodies being capable of binding a **Dengue** virus viral protein, said method comprising immunizing said animal with at least one polypeptide according to any one of claims. . .

. . . as claimed in claim 19 which further comprises in vitro labeling one or more isolated antibodies capable of binding a **Dengue** viral protein.

L14 ANSWER 9 OF 15 USPATFULL on STN
1998:68530 Trova fowl pox virus recombinants comprising heterologous inserts.
Paoletti, Enzo, Delmar, NY, United States
Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States

Norton, Elizabeth R., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyons, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe Francis, Albany, NY, United States
Gettig, Russell Robert, Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5766599 19980616
APPLICATION: US 1995-458101 19950601 (8) <--
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.
AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.
CLM What is claimed is:

1. An attenuated virus having all the identifying characteristics of: a TROVAC fowlpox virus.

2. A virus which is TROVAC.

3. A vector which comprises the virus of claim 1.

4. A vector which comprises the virus of claim 2.

5. A virus as claimed in claim 2 further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the virus genome.

6. A virus as claimed in claim 5 wherein the exogenous DNA is selected from the group consisting of rabies virus, Hepatitis B virus, Japanese **encephalitis virus**, **yellow fever virus**, **Dengue** virus, measles virus, pseudorabies virus, Epstein-Barr virus, herpes simplex virus, human immunodeficiency virus, simian immunodeficiency virus, equine herpes virus, bovine herpes virus, bovine viral diarrhea virus, human cytomegalovirus, canine parvovirus, equine influenza virus, feline leukemia virus, feline herpes virus, Hantaan virus, C. tetani, avian influenza virus, mumps virus and Newcastle Disease virus.

7. A virus as claimed in claim 6 wherein the non-poxvirus source is avian influenza virus and the fowlpox virus is vFP89, vFP92, vFP100 or vFP122.

8. A virus as claimed in claim 6 wherein the virus is a fowlpox virus, the non-poxvirus source is human immunodeficiency virus and the fowlpox virus is vFP62, vFP63 or vFP174.

9. A virus as claimed in claim 6 wherein the non-poxvirus source is Newcastle Disease virus and the fowlpox virus is vFP96.

10. A virus as claimed in claim 6 which is a human immunodeficiency virus recombinant fowlpox virus which is vFP62 or vFP63.

11. A virus as claimed in claim 1 further comprising exogenous DNA from a non-poxvirus source in a nonessential region of the virus genome.

12. An immunological composition for inducing an immunological response in a host animal inoculated with said composition, said composition comprising the virus of any one of claims 1, 2 or 10 or 11, or, a vector as claimed in claim 3 or 4, and a carrier.

13. The immunological composition of claim 12 containing the virus or vector in an amount sufficient to induce a protective immunological response such that the immunological composition is a vaccine.

14. A method of expressing a gene product in a cell cultured in vitro comprising introducing into the cell a virus as claimed in any one of claims 1, 2 or 10 or 11, or, a vector as claimed in claim 3 or 4, transforming cell with the expression vector, cultivating the transformed cell under conditions which allow expression of the gene product, and further purifying the product.

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SUMM . . . sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an **E**. coli plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the. . . flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within **E**. coli bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1982).

SUMM Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, **e**.g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively. . .

SUMM Cowpox virus (Brighton red strain) produces red (hemorrhagic) pocks on the chorioallantoic **membrane** of chicken eggs. Spontaneous deletions within the cowpox genome generate mutants which produce white pocks (Pickup et al., 1984). The. . .

DRWD FIG. 26 shows the nucleotide sequence of FeLV-B **Envelope** Gene (SEQ ID NO:310);

DETD . . . from Bethesda Research Laboratories, Gaithersburg, Md., New England Biolabs, Beverly, Mass.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind. Klenow fragment of **E**. coli polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England. . .

DETD . . . NcoI site (pos. 172,253) were removed by digestion of pSD419 with NcoI/SmaI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation generating plasmid pSD476. A vaccinia right flanking arm was obtained by digestion of pSD422 with HpaI. . . ID NO:6/SEQ ID NO:7) ##STR3## generating pSD479. pSD479 contains an initiation codon (underlined) followed by a BamHI site. To place **E**. coli Beta-galactosidase in the B13-B14 (u) deletion locus under the control of the u promoter, a 3.2 kb BamHI fragment. . .

DETD . . . at the pUC/vaccinia junction was destroyed by digestion of pSD478 with EcoRI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation, generating plasmid pSD478E$^-$. pSD478E$^-$ was digested with BamHI and HpaI and ligated with annealed synthetic oligonucleotides. . .

DETD . . . XbaI within vaccinia sequences (pos. 137,079) and with HindIII at the pUC/vaccinia junction, then blunt ended with Klenow fragment of **E**. coli polymerase and ligated, resulting in plasmid pSD483. To remove unwanted vaccinia DNA sequences to the right of the A26L. . . digestion with BglII (pos. 140,136) and with EcoRI at the pUC/vaccinia junction, followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation. The resulting plasmid was designated pSD489. The 1.8 kb ClaI (pos. 137,198)/EcoRV (pos. 139,048) fragment from. . .

DETD A 3.3 kb BglII cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD A 3.2 kb BglII/BamHI (partial) cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD . . . were removed from the pUC/vaccinia junction by digestion of pSD466 with EcoRI/BamHI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation. Recombination between vP708 and pSD467 resulted in recombinant vaccinia deletion mutant, vP723, which was isolated as. . .

DETD . . . SphI and religated, forming pSD451. In pSD451, DNA sequences to the left of the SphI site (pos. 27,416) in HindIII **M** are removed (Perkus et al., 1990). pSD409 is HindIII **M** cloned into pUC8.

DETD To provide a substrate for the deletion of the [C7L-K1L] gene cluster from vaccinia, **E**. coli Beta-galactosidase was first inserted into the vaccinia M2L deletion locus (Guo et al., 1990) as follows. To eliminate the. . . unique BglII site inserted into the M2L deletion locus as indicated above. A 3.2 kb BamHI (partial)/BglII cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the 11 kDa promoter (Bertholet et al., 1985) was. . .

DETD . . . deleted for vaccinia genes [C7L-K1L] was assembled in pUC8 cut with SmaI, HindIII and blunt ended with Klenow fragment of **E**. coli polymerase. The left flanking arm consisting of vaccinia HindIII C sequences was obtained by digestion of pSD420 with XbaI (pos. 18,628) followed by blunt ending with Klenow fragment of **E**. coli polymerase and digestion with BglII (por. 19,706). The right flanking arm consisting of vaccinia HindIII K sequences was obtained. . .

DETD . . . coding sequences, pSD518 was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001) and blunt ended using Klenow fragment of **E**. coli polymerase. This 4.8 kb vector fragment was ligated with a 3.2 kb SmaI cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD . . . al., 1989) was inserted into pCE13 by digesting pCE13 with SalI, filling in the sticky ends with Klenow fragment of **E**. coli DNA polymerase and digesting with HindIII. A HindIII-EcoRV fragment containing the H6 promoter sequence was then inserted into pCE13. . .

DETD . . . H6 promoted NDV-P cassette by cloning a HindIII fragment from pCE59 that had been filled in with Klenow fragment of **E**. coli DNA polymerase into the HpaI site of pCE71 to form pCE80. Plasmid pCE80 was completely digested with NdeI and. . .

DETD In NDV-infected cells, the F glycoprotein is anchored in the **membrane** via a hydrophobic transmembrane region near the carboxyl terminus and requires post-translational cleavage of a precursor, F_0, into two disulfide. . .

DETD . . . that immunoreactive proteins were presented on the infected cell surface. To determine that both proteins were presented on the plasma **membrane**, mono-specific rabbit sera were produced against vaccinia recombinants expressing either the F or HN glycoproteins. Indirect immunofluorescence using these sera. . .

DETD . . . mutagenized expression cassette contained within pRW837 was derived by digestion with HindIII and EcoRI, blunt-ended using the Klenow fragment of **E**. coli DNA polymerase in the presence of 2 mM dNTPs, and inserted into the SmaI site of pSD513 to yield. . .

DETD . . . into pRW843 (containing the measles HA gene). Plasmid pRW843 was first digested with NotI and blunt-ended with Klenow fragment of **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The resulting plasmid, pRW857, therefore contains the measles virus F. . .

DETD Immunoprecipitation. Immunoprecipitation reactions were performed as previously described (Taylor et al., 1990) using a guinea-pig anti measles serum (Whittaker **M**. A. Bioproducts, Walkersville, Md.).

DETD . . . mutagenesis was done using MRSYN5 (SEQ ID NO:52) (5'-GCGAGCGAGGCCATGC ATCGTGCGAATGGCCCC-3') and MRSYN6 (SEQ ID NO:53) (5'-GGGGG GACGCGCGGGTCTAGAAGGCCCCGCCTGGCGG-3') and selection on **E**. coli dut$^-$ ung$^-$ strain. CJ236 (International Biotechnologies, Inc., New Haven, Conn.). Mutagenesis was performed according to the protocols of Kunkel. . .

DETD . . . A 1.4 kb fragment containing the I3L promoter/PRV gp50 gene was isolated and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs.

DETD Immunoprecipitation from NYVAC/PRV Recombinant Infected Cells. Vero

cells were infected at an **m**.o.i. of 10 pfu per cell with the individual recombinant viruses, with the NYVAC parent virus, or were mock infected. After. . . were dissociated with RIPA buffer (1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 0.01M methionine, 5 mM EDTA, 5 MM 2-mercapto-ethanol, 1 **m**/ml BSA, and 100 u/ml aprotinin). Samples analyzed with sheep anti-gplII and a monoclonal specific for gp50 were lysed in 1X. . .

DETD Extraneous 3'-noncoding sequence was then eliminated from pGC10. This was accomplished by recircularizing the **E**. coli DNA polymerase I (Klenow fragment) filled-in 4,900 bp SalI-SmaI (partial) fragment of pGC10. The plasmid generated by this manipulation. . .

DETD Extraneous DNA was then eliminated. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 6,000 bp HindIII-BamHI (partial) fragment of pGBCD1, containing the H6-promoted gB, gC and. . .

DETD Immunoprecipitation. Vero cells were infected at an **m**.o.i. of 10 pfu per cell with recombinant vaccinia virus, with the NYVAC parent virus (vP866) or were mock infected. After. . .

DETD . . . inserted individually into three different sites of the virus. The three HBV genes encode the following protein products: (1) HBV **M** protein, (referred to here as small pre S antigen, or spsAg), (2) HBV L protein (referred to here as large. . .

DETD The synthetic S1+S2 region was assembled in five double stranded sections A through **E** as indicated above using synthetic oligonucleotides, MPSYN290 through MPSYN308 (SEQ ID NO:90)-(SEQ ID NO:99), as set out below. Oligonucleotides ranged. . . within a section were kinased before annealing of the section. Sequence of synthetic oligonucleotides used to construct sections A through **E** are given below. Only the coding strand is shown. Relevant restriction sites are noted. Initiation codons for S1 (section A), S2 (section C) and core (section **E**) are underlined. ##STR19## The vaccinia I3L promoter was synthesized using pMP1, a subclone of HindIII I, as template and synthetic. . .

DETD Construction of Insertion Vector Containing the Rabies G Gene. Construction of pRW838 is illustrated below. Oligonucleotides A through **E**, which overlap the translation initiation codon of the H6 promoter with the ATG of rabies G, were cloned into pUC9 as pRW737. Oligonucleotides A through **E** contain the H6 promoter, starting at NruI, through the HindIII site of rabies G-followed by BglII. Sequences of oligonucleotides A through **E** (SEQ ID NO:109)-(SEQ ID NO. 113) are: ##STR22## The diagram of annealed oligonucleotides A through **E** is as follows: ##STR23##

DETD Oligonucleotides A through **E** were kinased, annealed (95° C. for 5 minutes, then cooled to room temperature), and inserted between the PvuII sites of. . .

DETD . . . stages of assembly of mature rabies virus particles, the glycoprotein component is transported from the golgi apparatus to the plasma **membrane** where it accumulates with the carboxy terminus extending into the cytoplasm and the bulk of the protein on the external surface of the cell **membrane**. In order to confirm that the rabies glycoprotein expressed in ALVAC-RG was correctly presented, immunofluorescence was performed on primary CEP. . .

DETD The initial inoculation was performed at an **m**.o.i. of 0.1 pfu per cell using three 60 mm dishes of each cell line containing 2×10^6 cells per dish. One. . .

DETD . . . parental canarypox virus, (b) ALVAC-RG, the recombinant expressing the rabies G glycoprotein or (c) vCP37, a canarypox recombinant expressing the **envelope** glycoprotein of feline leukemia virus. Inoculations were performed under ketamine anaesthesia. Each animal received at the same time: (1) 20. . .

DETD (**e**) Primary CEF cells.

DETD . . . electrophoresis the viral DNA band was visualized by staining with ethidium bromide. The DNA was then transferred to a nitrocellulose **membrane** and probed with a radiolabelled probe prepared from purified ALVAC genomic DNA.

Vaccine Batch H		
	23	4.52
Vaccine Batch I		
	23	3.33
Vaccine Batch K		
	15	3.64
Vaccine Batch L		
	15	4.03
Vaccine Batch M		
	15	3.32
Vaccine Batch N		
	15	3.39
Vaccine Batch J		
	23	3.42

[a] Expressed as mouse LD_{50}

[b] Expressed as $\log_{10}$ $TCID_{50}$

DETD . . . 2.2

2.2

2.2

39 vCP37[d]

NT <1.2

<1.2

1.7

2.1

2.2

N.T.[g]

55 vCP37[d]

NT <1.2

<1.2

1.7

2.2

2.1

N.T.

37 ALVAC-RG[e]

2.2 <1.2

<1.2

3.2

3.5

3.5

3.2

53 ALVAC-RG[e]

2.2 <1.2

<1.2

3.6

3.6

3.6

3.4

38 ALVAC-RG[f]

2.7 <1.7

<1.7

3.2

3.8

3.6

N.T.

54 ALVAC-RG[f]

3.2 <1.7

<1.5

3.6

. . . 28 after primary vaccination

[c] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of ALVAC

[d] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of vCP37

[e] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of ALVACRG

[f] Animals received 7.0 $\log_{10}$ $TCID_{50}$ of ALVACRG

[a] Not tested.

DETD TABLE 15

Inoculation of chimpanzees with ALVAC-RG

Weeks post-Inoculation	Animal 431 I.M.	Animal 457 S.C.
0	<8[a]	<8
1	<8	<8
2	8	32
4	16	32
8	16	32
12[b] /0	16	8
13/1	128	128
15/3	256	512
20/8.	. .	

DETD Construction of NYVAC Recombinants Expressing **Flavivirus** Proteins

DETD This example describes the construction of NYVAC donor plasmids containing genes from Japanese **encephalitis virus** (JEV), **yellow fever virus** (YF) and **Dengue** type 1, the isolation of the corresponding NYVAC **Flavivirus** recombinants and the ability of vaccinia recombinants expressing portions of the genomes of JEV or YF to protect mice against. . .

DETD . . . and AccI fragment of JEV2 (Mason et al., 1991) containing the plasmid origin and JEV cDNA encoding the carboxy-terminal 40% **prM** and amino-terminal two thirds of **E** (nucleotides 602 to 2124), generating plasmid JEV20 containing JE sequences from the ATG of C through the SacI site (nucleotide 2124) found in the last third of **E**.

DETD . . . 1991) in which TTTTTGT nucleotides 1304 to 1310 were changed to TCTTTGT), containing JE sequences from the last third of **E** through the first two amino acids of NS2B (nucleotides 2124 to 4126), the plasmid origin and vaccinia sequences, was ligated. . .

DETD . . . end] generated plasmid JEV25 which contains JE cDNA extending from the SacI site (nucleotide 2124) in the last third of **E** through the carboxy-terminus of **E**. The SacI-EagI fragment from JEV25 was ligated to the SacI-EagI fragment of JEV8 (containing JE cDNA encoding 15 aa C, **prM** and amino-terminal two thirds of **E** nucleotides 337 to 2124, the plasmid origin and vaccinia sequences) yielding plasmid JEV26. A unique SmaI site preceding the ATG. . .

DETD . . . fragment from JEV7 (Mason et al., 1991) yielded JEV29 (containing a SmaI site followed by JE cDNA encoding 30 aa **E**, NS1, NS2A nucleotides 2293 to 4126) and JEV30 (containing a SmaI site followed by JE cDNA encoding 30 aa **E**, NS1, NS2A, NS2B nucleotides 2293 to 4512).

DETD . . . Vitro Virus Infection and Radiolabeling. HeLa cell monolayers were prepared in 35 mm diameter dishes and infected with vaccinia viruses (**m**.o.i. of 2 pfu per cell) or JEV (**m**.o.i. of 5 pfu per cell) before radiolabeling. Cells were pulse labeled with medium containing ^{35}S-Met and chased for 6. . .

DETD Recombinant vP825 encoded the capsid protein, structural protein precursor **prM**, the structural glycoprotein **E**, the nonstructural glycoprotein NS1, and the nonstructural protein NS2A (McAda et al., 1987). Recombinant vP829 encoded the putative 15 aa **signal sequence** preceding the amino-terminus of **prM**, as well as **prM**, and **E** (McAda et al., 1987). Recombinant vP857 contained a cDNA encoding the 30 aa hydrophobic carboxy-terminus of **E**, followed by NS1 and NS2A. Recombinant vP864 contained a cDNA encoding the same proteins as vP857 with the addition of NS2B. In recombinants vP825 and vP829 a potential vaccinia virus early transcription termination signal in **E** (TTTTTGT; nucleotides 1304-1310) was modified to TCTTTGT without altering the aa sequence. This change was made in an attempt to increase the level of expression of **E** since this sequence has been shown to increase transcription termination in in vitro transcription assays (Yuen et al., 1987).

DETD **E** and **prM** Are Correctly Processed When Expressed By Recombinant

vaccinia viruses. Pulse chase experiments demonstrate that proteins identical in size to **E** were synthesized in cells infected with all recombinant vaccinia viruses containing the **E** gene (Table 16). In the case of cells infected with JEV, vP555 and vP829, an **E** protein that migrated slower in SDS-PAGE was also detected in the culture fluid harvested from the infected cells (Table 16). This extracellular form of **E** produced by JEV- and vP555-infected cells contained mature N-linked glycans (Mason, 1989; Mason et al., 1991), as confirmed for the extracellular forms of **E** produced by vP829-infected cells. Interestingly, vP825, which contained the C coding region in addition to **prM** and **E** specified the synthesis of **E** in a form that is not released into the extracellular fluid (Table 16). Immunoprecipitations prepared from radiolabeled recombinant vaccinia-infected cells using a MAb specific for **M** (and **prM**) revealed that **prM** was synthesized in cells infected with vP555, vP825, and vP829, and **M** was detected in the culture fluid of cells infected with vP555 or vP829 (Table 16).

DETD . . . (data not shown). This result indicated that vP829 infected cells produce extracellular particles similar to the empty viral envelopes containing **E** and **M** observed in the culture fluids harvested from vP555 infected cells (Table 16 and Mason et al., 1991).

DETD . . . To JEV Antigens. Pre-challenge sera pooled from selected animals in each group were tested for their ability to immunoprecipitate radiolabeled **E** and NS1. The results of these studies (Table 16) demonstrated that: (1) the magnitude of immune response induced to **E** was vP829>vP555>vP825, (2) all viruses encoding NS1 and NS2A induced antibodies to NS1, and (3) all immune responses were increased. . . sera collected from these animals (Table 17) confirmed the results of the immunoprecipitation analyses, showing that the immune response to **E** as demonstrated by RIP correlated well with these other serological tests (Table 17).

DETD TABLE 16

Characterization of proteins expressed by vaccinia recombinants expressing JEV proteins and their immune responses

	vP555	vP829	vP825	vP857	vP864
Proteins expressed[a]					
Intracellular	**prM, E** NS1	**prM, E** NS1	**prM, E** NS1	NS1	NS1
secreted	**M, E**, NS1	**M,E**	none	NS1	NS1
Particle formation[b]	+	+	-	-	-
Immune response[c]					
single	**E**	**E**	NS1	NS1	NS1
double	**E**, NS1	**E**	**E**, NS1	NS1	NS1

[a] Radiolabelled cell lysates and culture fluids from vaccinia virus JEV recombinant infected cells were harvested and JEVspecific proteins immunoprecipitated using mAbs to **E**, **M** and NS1 proteins.

[b] Formation of extracellular particles with HA activity as described in the text.

[c] JEV proteins were. . .

DETD . . . isolated and ligated to a SacI (JEV nucleotide 2124) to EagI fragment of JEV25 (containing the remaining two thirds of **E**, translation stop and T5NT) generating JEV36. JEV36 was transfected into vP866 (NYVAC) infected cells to generate the vaccinia recombinant vP923. . .

DETD Plasmid YF0 containing YF cDNA encoding the carboxy-terminal 80% **prM**, **E** and amino-terminal 80% NS1 (nucleotides 537-3266) was derived by cloning an AvaI to NsiI fragment of YF cDNA (nucleotides 537-1659). . . and KpnI digested IBI25 (International Biotechnologies, Inc., New Haven, Conn.). Plasmid YF1 containing YF cDNA encoding C and

amino terminal 20% **prM** (nucleotides 119-990) was derived by cloning a RsaI to AvaI fragment of YF cDNA (nucleotides 166-536) and annealed oligonucleotides SP46. . . and YF nucleotides 119-165) into AvaI and HindIII digested IBI25. Plasmid YF3 containing YF cDNA encoding the carboxy-terminal 60% of **E** and amino-terminal 25% of NS1 was generated by cloning an ApaI to BamHI fragment of YF cDNA (nucleotides 1604-2725) into. . . cDNA (nucleotides 4339-4940) into SacI and XbaI digested IBI25. Plasmid YF13 containing YF cDNA encoding the carboxy-terminal 25% of C, **prM** and amino-terminal 40% of **E** was derived by cloning a BalI to ApaI fragment of YF cDNA (nucleotides 384-1603) into ApaI and SmaI digested IBI25.

DETD . . . in YF1 (TTTTTCT nucleotides 263-269 and TTTTTGT nucleotides 269-275) to (SEQ ID NO:122) TTCTTCTTCTTGT creating plasmid YF1B, (2) in the **E** gene in YF3 (nucleotides 1886-1893 TTTTTTGT to TTCTTTGT 189 aa from the carboxy-terminus and nucleotides 2429-2435 TTTTTGT to TTCTTGT 8. . . YF3C (nucleotides 1965-2725) was exchanged for the corresponding fragment of YF3B generating YF4 containing YF cDNA encoding the carboxy-terminal 60% **E** and amino-terminal 25% NS1 (nucleotides 1604-2725) with both mutagenized transcription termination signals. An ApaI to BamHI fragment from YF4 (nucleotides 1604-2725) was substituted for the equivalent region in YF0 creating plasmid YF6 containing YF cDNA encoding the carboxy-terminal 80% **prM**, **E** and amino-terminal 80% NS1 (nucleotides 537-3266) with both mutagenized transcription termination signals. Plasmid YF6 was digested with EcoRV within the. . .

DETD . . . described above was used (1) to insert XhoI and ClaI sites preceding the ATG 17 aa from the carboxy-terminus of **E** (nucleotides 2402-2404) in plasmid YF3C creating YF5, (2) to insert XhoI and ClaI sites preceding the ATG 19 aa from the carboxy-terminus of **prM** (nucleotides 917-919) in plasmid YF13 creating YF14, (3) to insert an XhoI site preceding the ATG 23 aa from the carboxy-terminus of **E** (nucleotides 2384-2386) in plasmid YF3C creating plasmid YF25, (4) and to insert an XhoI site and ATG (nucleotide 419) in. . .

DETD . . . YF5 (nucleotides 1604-2725) was exchanged for the corresponding region of YF0 creating YF7 containing YF cDNA encoding the carboxy-terminal 80% **prM**, **E** and amino-terminal 80% NS1 (nucleotides 537-3266) with XhoI and ClaI sites at 2402 (17 aa from the carboxy-terminus of **E**) and a mutagenized transcription termination signal at 2429-2435 (8 aa from the carboxy-terminus of **E**). The ApaI to BamHI fragment from YF25 (nucleotides 1604-2725) was exchanged for the corresponding region of YF0 generating YF26 containing YF cDNA encoding the carboxy-terminal 80% **prM**, **E** and amino-terminal 80% NS1 (nucleotides 537-3266) with an XhoI site at nucleotide 2384 (23 aa from the carboxy-terminus of **E**) and mutagenized transcription termination signal at 2428-2435 (8 aa from the carboxy-terminus of **E**).

DETD . . . YF14 (nucleotides 537-1603) was substituted for the corresponding region in YF6 generating YF15 containing YF cDNA encoding the carboxy-terminal 80% **prM**, **E** and amino-terminal 80% NS1 (nucleotides 537-3266) with XhoI and ClaI sites at nucleotide 917 (19 aa from the carboxy-terminus of **prM**) and two mutagenized transcription termination signals. YF6 was digested within IBI25 with EcoRV and within YF at nucleotide 537 with. . .

DETD . . . from YF12 encoding carboxy-terminal 20% NS1, NS2A and NS2B (nucleotides 3267-4569), XhoI to KpnI fragment from YF15 encoding 19 aa **prM**, **E** and amino-terminal 80% NS1 (nucleotides 917-3266) and XhoI-SmaI digested pHES4 were ligated generating YF23. An XhoI to BamHI fragment from YF26 encoding 23 aa **E**, amino-terminal 25% NS1 (nucleotides 2384-2725) was ligated to an XhoI to BamHI fragment from YF23 (containing the carboxy-terminal 75% NS1,. . .

DETD XhoI-SmaI digested pHES4 was ligated to a purified XhoI to KpnI fragment from YF7 encoding 17 aa **E** and amino-terminal 80% NS1 (nucleotides 2402-3266) plus a KpnI to SmaI fragment from YF10 encoding the carboxy-terminal 20% NS1 and NS2A (nucleotides 3267-4180) creating YF18. An XhoI to BamHI fragment from YF2 encoding C, **prM**, **E** and amino-terminal 25% NS1 (nucleotides 119-2725) was ligated to a XhoI to BamHI fragment of YF18 (containing the carboxy-terminal 75%. . . the

origin of replication and vaccinia sequences) generating YF20. A XhoI to BamHI fragment from YF46 encoding 21 aa C, **prM**, **E** and amino-terminal 25% NS1 (nucleotides 419-2725) was ligated to the XhoI to BamHI fragment from YF18 generating YF47. Oligonucleotide SP46. . .

DETD Recombinant vP725 encoded the putative 17-aa **signal |sequence** preceding the N terminus of the nonstructural protein NS1 and the nonstructural proteins NS1 and NS2A (Rice et al., 1985). Recombinant vP729 encoded the putative 19-aa **signal sequence** preceding the N terminus of **E**, **E**, NS1, NS2A and NS2B (Rice et al., 1985). Recombinant vP764 encoded C, **prM**, **E**, NS1, NS2A and NS2B (Rice et al., 1985). Recombinant vP766 encoded C, **prM**, **E**, NS1 and NS2A (Rice et al., 1985). Recombinant vP869 encoded the putative 21-aa **signal sequence** preceding the N terminus of the **prM** structural protein precursor as well as **prM**, **E**, NS1 and NS2A (Rice et al., 1985).

DETD . . . NYVAC Donor Plasmid. A XhoI to AvaI fragment from YF47 (nucleotides 419-4180) containing YF cDNA encoding 21 amino acids C, **prM**, **E**, NS1, NS2A (with nucleotide 2962 missing in NS1) was ligated to XhoI-SmaI digested SPHA-H6 (HA region donor plasmid) generating YF48.. . . (nucleotide 3262) and a 6700 bp fragment isolated (containing the plasmid origin of replication, vaccinia sequences, 21 amino acids C, **prM**, **E**, amino-terminal 3.5% NS1, carboxy-terminal 23% NS1, NS2A) and ligated to a SacI-Asp718 fragment from YF18 (containing the remainder of NS1. . . in YF51 were removed using oligonucleotide-directed double-strand break mutagenesis (Mandecki, 1986) creating plasmid YF50 encoding YF 21 amino acids C, **prM**, **E**, NS1, NS2A in the HA locus donor plasmid. Donor plasmid YF50 was transfected into vP866 (NYVAC) infected cells to generate. . .

DETD . . . double-strand break mutagenesis creating YF49. Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to insert a SmaI site at the carboxy-terminus of **E** (nucleotide 2452) in YF4 creating YF16. An ApaI-SmaI fragment of YF49 (containing the plasmid origin of replication, vaccinia sequences and YF cDNA encoding 21 amino acid C, **prM**, and amino-terminal 43% **E**) was ligated to an ApaI-SmaI fragment from YF16 (nucleotides 1604-2452 containing the carboxy-terminal 57% **E**) generating YF53 containing 21 amino acids of C, **prM**, **E** in the HA locus. Donor plasmid YF53 was transfected into vP913 (NYVAC-MV) infected cells to generate the vaccinia recombinant vP997.

DETD Cloning of **Dengue** Type 1 Into a Vaccinia Virus Donor Plasmid. Plasmid DEN1 containing DEN cDNA encoding the carboxy-terminal 84% NS1 and amino-terminal. . . digested pUC8. An EcoRI-HindIII fragment from DEN1 (nucleotides 2559-3745) and SacI-EcoRI fragment of DEN cDNA encoding the carboxy-terminal 36% of **E** and amino-terminal 16% NS1 (nucleotides 1447-2559, Mason et al., 1987b) were ligated to HindIII-SacI digested IBI24 (International Biotechnologies, Inc., New Haven, Conn.) generating DEN3 encoding the carboxy-terminal 64% **E** through amino-terminal 45% NS2A with a base missing in NS1 (nucleotide 2467).

DETD . . . an AvaI-SacI fragment of DEN cDNA (nucleotides 424-1447 Mason et al., 1987) generating DEN4 encoding the carboxy-terminal 11 aa C, **prM** and amino-terminal 36% **E**.

DETD Plasmid DEN6 containing DEN cDNA encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 (nucleotides 1447-2579 with nucleotide 2467 present Mason et al., 1987b) was derived by cloning a SacI-XhoI. . . Biotechnologies, Inc., New Haven, Conn.). Plasmid DEN15 containing DEN cDNA encoding 51 bases of the DEN 5' untranslated region, C, **prM** and amino-terminal 36% **E** was derived by cloning a HindIII-SacI fragment of DEN cDNA (nucleotides 20-1447, Mason et al., 1987b) into HindIII-SacI digested IBI25.. . .

DETD . . . change the following potential vaccinia virus early transcription termination signals (Yuen et al., 1987). The two T5NT seqeunces in the **prM** gene in DEN4 were mutagenized (1) 29 aa from the carboxy-terminus (nucleotides 822-828 TTTTTCT to TATTTCT) and (2) 13 aa. . .

DETD . . . 4102) in plasmid DEN23 creating DEN24, (2) to insert a SmaI site and ATG 15 aa from the carboxy-terminus of **E** in DEN7 (nucleotide

2348) creating DEN10, (3) to insert an EagI and HindIII site at the carboxy-terminus of NS2B (nucleotide. . .

DETD . . . DEN7 (nucleotides 1447-2579) was substituted for the corresponding region in DEN3 generating DEN19 containing DEN cDNA encoding the carboxy-terminal 64% **E** and amino-terminal 45% NS2A (nucleotides 1447-3745) with nucleotide 2467 present and the modified transcription termination signal (nucleotides 2448-2454). A XhoI-XbaI. . .

DETD A HindIII-PstI fragment of DEN16 (nucleotides 20-494) was ligated to a HindIII-PstI fragment from DEN47 (encoding the carboxy-terminal 83% **prM** and amino-terminal 36% of **E** nucleotides 494-1447 and plasmid origin of replication) generating DEN17 encoding C, **prM** and amino-terminal 36% **E** with part of the H6 promoter and EcoRV site preceding the amino-terminus of C. A HindIII-BglII fragment from DEN17 encoding the carboxy-terminal 13 aa C, **prM** and amino-terminal 36% **E** (nucleotides 370-1447) was ligated to annealed oligonucleotides SP111 and SP112 (containing a disabled HindIII sticky end, EcoRV site to -1. . . a BglII sticky end) creating DEN33 encoding the EcoRV site to -1 of the H6 promoter, carboxy-terminal 20 aa C, **prM** and amino-terminal 36% **E**.

DETD . . . digested pTP15 (Mason et al., 1991) was ligated to a SmaI-SacI fragment from DEN4 encoding the carboxy-terminal 11 aa C, **prM** and amino-terminal 36% **E** (nucleotides 377-1447) and SacI-EagI fragment from DEN3 encoding the carboxy-terminal 64% **E**, NS1 and amino-terminal 45% NS2A generating DENL. The SacI-XhoI fragment from DEN7 encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 (nucleotides 1447-2579) was ligated to a BstEII-SacI fragment from DEN47 (encoding the carboxy-terminal 55% **prM** and amino-terminal 36% **E** nucleotides 631-1447) and a BstEII-XhoI fragment from DENL (containing the carboxy-terminal 11 aa C, amino-terminal 45% **prM**, carboxy-terminal 82% NS1, carboxy-terminal 45% NS2A, origin of replication and vaccinia sequences) generating DEN8. A unique AvaI site (located between. . .

DETD . . . an EcoRV-SacI fragment of DEN8VC (containing vaccinia sequences, H6 promoter from -21 to -124, origin of replication and amino-terminal 64% **E**, NS1, amino-terminal 45% NS2A nucleotides 1447-3745) generating DEN18. A XhoI-EagI fragment from DEN25 (nucleotides 2579-4102) was ligated to an XhoI-EagI fragment of DEN18 (containing the origin of replication, vaccinia sequences and DEN C, **prM**, **E** and amino-terminal 18% NS1 nucleotides 68-2579) generating DEN26. An EcoRV-SacI fragment from DEN8VC (positions -21 to -1 H6 promoter DEN. . . to an EcoRV-SacI fragment of DEN26 (containing the origin of replication, vaccinia sequences and DEN region encoding the carboxy-terminal 64% **E**, NS1 and NS2A with a base missing in NS1 at nucleotide 2894) generating DEN32. DEN32 was transfected into vP410 infected. . .

DETD . . . DEN10 (nucleotides 1447-2579) was substituted for the corresponding region in DEN3 generating DEN11 containing DEN cDNA encoding the carboxy-terminal 64% **E**, NS1 and amino-terminal 45% NS2A with a SmaI site and ATG 15 aa from the carboxy-terminus of **E**. A SmaI-EagI fragment from DEN11 (encoding the carboxy-terminal 15 aa **E**, NS1 and amino-terminal 45% NS2A nucleotides 2348-3745) was ligated to SmaI-EagI digested pTP15 generating DEN12.

DETD An EcoRV-XhoI fragment from DEN8VC (positions -21 to -1 H6 promoter DEN nucleotides 377-2579 encoding the carboxy-terminal 11 aa C, **prM E**, amino-terminal 18% NS1) was ligated to an EcoRV-XhoI fragment from DEN31 (containing the origin of replication, vaccinia sequences and DEN. . . An EcoRV-SacI fragment from DEN33 (positions -21 to -1 H6 promoter DEN nucleotides 350-1447 encoding the carboxy-terminal 20 aa C, **prM** and amino-terminal 36% **E**) and a SacI-XhoI fragment from DEN32 (encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 nucleotides 1447-2579) were ligated to the EcoRV-SacI fragment from DEN31 described above generating DEN34. DEN34 was. . .

DETD . . . the left terminus of vaccinia and by introducing a deletion near the right terminus. All deletions were accomplished using the **E**. coli guanine phosphoribosyl transferase gene and mycophenolic acid in a transient selection system.

DETD For use as a selectable marker, the **E**. coli gene encoding guanine phosphoribosyl transferase (Ecogpt) (Pratt at al., 1983) was placed under the control of a poxvirus promoter.. . .

DETD . . . subunit of ribonucleotide reductase (Slabaugh et al., 1988). Also included in this deletion is ORF F2L, which shows homology to **E**. coli dUTPase, another enzyme involved in nucleotide metabolism (Goebel et al., 1990a,b). F2L also shows homology to retroviral protease (Slabaugh. . .

DETD . . . sequences, the predicted translation product of Copenhagen ORF B16 is truncated at the amino terminus and does not contain a **signal sequence**. B19R encodes a vaccinia surface protein (S antigen) expressed at early times post infection (Ueda et al., 1990). Both B16R. . .

DETD . . . immunological assays was comprised of RPMI 1640 medium supplemented with 10% FBS, 4 uM L-glutamine, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate), 5×10^{-5} **M** 2-mercaptoethanol, 100 IU penicillin per ml, and 100 μg/ml streptomycin. Stim Medium was comprised of Eagle's Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, 10^{-4} **M** 2-mercaptoethanol, 100 IU penicillin per ml, and 100 μg streptomycin per ml.

DETD ALVAC and NYVAC Recombinants Containing the V3 Loop and Epitope 88 of the HIV-1 (IIIB) **Envelope**. A 150 bp fragment encompassing the V3 loop (amino acids 299-344; Javeherian et al., 1989) of HIV-1 (IIIB) was derived. . .

DETD . . . isolated by phenol extraction (2×) and ether extraction (1×). The isolated fragment was blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The fragment was ligated to pSD550, a derivative of pSD548. . .

DETD ALVAC- and NYVAC-Based Recombinants Expressing the HIV-1 (IIIB) **Envelope** Glycoproteins. An expression cassette composed of the HIV-1 (IIIB) env gene juxtaposed 3' to the vaccinia virus H6 promoter (Guo. . .

DETD . . . pBSHIV3BEAII was digested with NruI and XbaI. The derived 2.7 kb fragment was blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This fragment contains the entire HIV-1 env gene juxtaposed 3'. . .

DETD . . . followed by a partial KpnI digestion. The 1.6 kb fragment was blunt-ended by treatment with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This fragment was inserted into pSD54IVC digested with SmaI to. . .

DETD . . . Vero cells monolayers were either mock infected, infected with the parental virus vP866, or infected with recombinant virus at an **m**.o.i. of 10 PFU/cell. Following a 1 hr adsorption period, the inoculum was aspirated and the cells were overlayed with 2. . .

DETD . . . using sera pooled from HIV-1 seropositive individuals showed specific precipitation of the gp120 and gp41 mature forms of the gp160 **envelope** glycoprotein from vP911 infected cell lysates. No such specific gene products were detected in the parentally (NYVAC; vP866) infected cell. . .

DETD . . . for 1 hour in tissue culture medium containing 2% FBS at 37° C. with the appropriate vaccinia virus at a **m**.o.i. of 25 pfu per cell. Following infection, the stimulator cells were washed several times in Stim Medium and diluted to. . .

DETD . . . cells were infected overnight by incubation at 1×10^{7} cells per ml in tissue culture medium containing 2% FBS at a **m**.o.i. of 25 pfu per cell for 1 hour at 37° C. Following incubation, the cells were diluted to between $1\text{-}2\times10^{6}$. . .

DETD . . . 1.8 2.2 1.2

vP911		-4.0	4.6 *	1.4	
	±	2.5	2.0	5.1	
vP921		-3.4	10.7 *		
				15.5 *	
	±	0.9	1.5	2.8	

E:T = 100:1

P < 0.05 vs appropriate controls, Student's ttest

DETD Recombinant plasmid pIBI25env was used to transform competent **E**. coli CJ236 (dut- ung-) cells. Single-stranded DNA was isolated from phage derived by infection of the transformed **E**. coli CJ236 cells with the helper phage, MG408. This single-stranded template was used in vitro mutagenesis reactions (Kunkel et al.,. . .

DETD . . . 2.5 kb (envIS+) and 2.4 kb (envIS-), respectively, were isolated and blunt-ended by reaction with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mMdNTPs. These fragments were ligated with the 3.5 kb fragment derived by digestion of pSIVenvVV with NruI and PstI with a subsequent blunting step with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The plasmid pSIVenvVV contains the SIV env gene expression cassette. . .

DETD . . . seropositive individuals were performed as described in Materials and Methods. All six recombinants directed the synthesis of the HIV-1 gp161 **envelope** precursor. The efficiency of processing of gp160 to gp120 and gp41, however, varied between cell types and was also affected. . .

DETD . . . to yield pBSH6HIV2ENV. The 2.7 kb HindIII/XbaI insert from pBSH6HIV2ENV was isolated and blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTP. The blunt-ended fragment was inserted into a SmaI digested pSD5HIVC. . .

DETD . . . gp160. Vero cell monolayers were either mock infected, infected with the parental virus vP866, or infected with vP920 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD Human sera from HIV-2 seropositive individuals specifically precipitated the HIV-2 gp160 **envelope** glycoprotein from vP920 infected cells. Furthermore, the authenticity of the expressed HIV-2 env gene product was confirmed, since the gp160. . .

DETD . . . coding sequence juxtaposed 3' to the vaccinia virus H6 promoter. This fragment was blunted with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The blunt-ended fragment was ligated to SmaI digested pSDSHIVC to. . .

DETD . . . digestion with HindIII liberated a 2.7 kb HindIII/EcoRI fragment. This fragment was blunt-ended by treatment with Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The fragment was ligated into pSD550VC digested with SmaI. The. . .

DETD Expression Analyses. The SIV gp140 env gene product is a typical glycoprotein associated with the plasma **membrane** of infected cells. It is expressed as a polyprotein of 140 kDa that is proteolytically cleaved to an extracellular species. . .

DETD . . . and gag) in Vero cells infected with the NYVAC/HIV recombinants was analyzed by immunoprecipitation. Vero cells were infected at an **m**.o.i. of 10 with the individual recombinant viruses, with the NYVAC parent virus, or were mock infected. After a 1 hour. . .

DETD The plasmid pF7D3 was linearized with XhoI and blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This linearized plasmid was ligated with annealed oligonucleotides F7MCSB (SEQ. . .

DETD . . . the H6 promoter) and PstI. The 3.5 kb resultant fragment was isolated and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This blunt-ended fragment was ligated to a 1700 bp EcoRV/HpaI. . .

DETD . . . HA molecule is synthesized and glycosylated as a precursor molecule at the rough endoplasmic reticulum. During passage to the plasma **membrane** it undergoes extensive post-translational modification culminating in proteolytic cleavage into the disulphide linked HA_1 and HA_2 subunits and insertion into the host cell **membrane** where it is subsequently incorporated into mature viral envelopes. To determine whether the HA molecules produced in cells infected with. . .

DETD . . . 3'end-EcoRV fragment (D). Plasmid pVHAH6g13 was digested with BglII and KpnI to isolate the 1330 bp BglII-H6-EHV-1 gC 5'-KpnI fragment (**E**).

DETD Fragments C, D and E were finally ligated together into vector pSD541VC digested with BglII and XhoI to produce plasmid pJCA042. Plasmid pJCA042 is the. . .

DETD . . . region-BamHI fragment (L). Plasmid pVHAH6g13 was digested with BglII and XhoI to isolate the 440 bp BglII-H6-EHV-1 gC 5'-portion-XhoI fragment (**M**). Fragments K, L and **M** were then ligated together to produce plasmid pJCA040.

DETD . . . authentic BHV1 gIV glycoprotein. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1051 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gene was then cloned into pIBR8. This was accomplished by cloning the 2,285 bp StuI fragment of pIBRS6 into the **E**. coli DNA polymerase I (Klenow fragment) filled-in 4,300 bp StuI-BglII (partial) fragment of pIBR8. The plasmid generated by this manipulation. . .

DETD The H6-promoted BHV1 gI gene was then moved to a vaccinia virus donor plasmid. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 2,900 bp BglII-NcoI (partial) fragment of pIBR20 into the SmaI site of pSD542.. . .

DETD . . . gI and gIV glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1074 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . authentic BHV1 gIII glycoprotein. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1073 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gIII and gIV glycoproteins. Vero cell monolayers were either sock infected, infected with NYVAC or infected with vP1083 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gI and gIII glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1087 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gIII and gIV glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1079 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1 (obtained from Eurogentec, Liege, Belgium; Renard et al., European Patent Application No:86870095) with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . gE1 and gE2 glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP972 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . cloned into pIBI25. This was accomplished by blunt-ending the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1, containing the gE1 "gene", with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . cloned into pIBI25. This was accomplished by blunt-ending the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1, containing the gE1 "gene", with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . pig polyclonal serum followed by fluorescein isothiocyanate goat anti-guinea pig. Cells infected with vP1001 showed gB expressed on the plasma **membrane**. Weak internal expression was detected within cells infected with vCP139.

DETD . . . gene was excised from pED3 with NruI and XhoI and the purified fragment was cloned into pVQH6CP3L (plasmid described in **Flavivirus** section) cut with NruI and XhoI. The resulting plasmid, pC3-VP2, contains the H6 promoted VP2 gene flanked by the C3. . .

DETD . . . with EcoRI, which recognizes a unique EcoRI site within the canarypox sequences, and blunt-ended using the Klenow fragment of the

E. coli DNA polymerase. The resultant plasmid was designated as pCPCV1. This plasmid contains the vaccinia virus H6 promoter followed by. . .

DETD An M13 clone containing the hemagglutinin (HA) gene from equine influenza virus (A2/Suffolk/89) was provided by Dr. M. Binns (Animal Health Trust, P.O. Box 5, Newmarket, Suffolk, CB8 7DW, United Kingdom). This clone contains a full-length 1.7 kb. . .

DETD . . . recombination tests with vP425 as the rescuing virus to construct a recombinant vaccinia virus (vP453) which expresses the entire FeLV **envelope** glycoprotein.

DETD . . . tests with vP410 as the rescuing virus to generate vP456. This vaccinia virus recombinant was generated to express the entire **envelope** glycoprotein lacking the putative immunosuppressive region.

DETD . . . of the H6 promote sequence. The PstI site is located 420 bp downstream from the translation termination signal for the **envelope** glycoprotein open reading frame.

DETD . . . of the H6 promoter sequence. The HpaI site is located 180 bp downstream from the translation termination signal for the **envelope** glycoprotein open reading frame. These isolated fragments were blunt-ended. These 2.2 kbp H6/FeLV env sequences were inserted into the nonessential. . .

DETD . . . with EcoRI, which recognizes a unique EcoRI site within the canarypox sequences, and blunt-ended using the Klenow fragment of the E. coli DNA polymerase. The resultant plasmid was designated as pCPCV1. This plasmid contains the vaccinia virus H6 promoter followed by. . .

DETD The putative immunosuppressive region is situated within the p15E transmembrane region of the FeLV **envelope** glycoprotein (Cianciolo et al., 1986; Mathes et al., 1978). This region was deleted in the following manner. The FeLV-A env. . .

DETD . . . into the SmaI site of pSD553. This insertion was performed following blunt-ending the fragment with the Klenow fragment of the E. coli DNA polymerase in the presence of 2 mM dNTPs.

DETD Immunoprecipitation. Vero cell monolayers were infected at an m.o.i. equal to 10 pfu/cell with parental or recombinant viruses. At 1 hr post-infection, the inoculum was aspirated and methionine-free medium. . .

DETD In order to determine whether the env gene product expressed by vCP83 and vCP87 was transported to the plasma **membrane** of infected cells, immunofluorescence experiments were performed as described previously (Taylor et al., 1990). Primary CEF monolayers were infected with. . .

DETD . . . to challenge with feline leukemia virus

Group		Time (weeks) relative to challenge Cat No.		-5 E[1]	-2 V[2]	0	+3	+6	+9	+12
					EV	EV	EV	F[3] EV	FEV	FEV
1.	vCP 93: Felv-A		1	--	--	--	++	-++	+++	+++
			2.	.	.	++	-++	+++	+++	
			17	--	--	--	++	-++	+++	+++
			18	--	--	--	++	-++	+++	+++

*E = FeLV p27 antigen in plasma (ELISA)
V = infectious virus in plasma (virus isolation)
F = FeLV antigen in. . .

DETD The FHV-1 C0 strain genomic DNA was completely digested with EcoRI and the fragment M (4470 bp) was excised from the agarose gel (Geneclean procedure) and cloned into vector pBS-SK+ digested with EcoRI and phosphatased. The resulting plasmid containing the FHV-1 EcoRI M fragment was designated pHFeM2. The FHV-1 EcoRI M fragment complete nucleotide sequence for both strands was obtained from several subclones of the FHV-1 EcoRI M fragment inserted into vector pBS-SK+, using the modified T7 enzyme Sequenase (U.S. Biochemical Corp.) (Tabor and Richardson, 1987). Standard dideoxynucleotide chain termination reactions (Sanger et al., 1977) were performed using double-stranded

templates that had been denatured in 0.4 **M** NaOH (Hattori and Sakaki, 1986). The M13 forward and reverse primers were used to obtain the initial sequence of each clone.. . .

DETD . . . the FHV-1 gD 5'-most region were confirmed by direct sequencing of pJCA071. Plasmid pJCA067 is a subclone of FHV-1 EcoRI **M** fragment. It has been generated as follows. Plasmid pHFeM2 was digested with BamHI and the 1850 bp BamHI-BamHI fragment was. . .

DETD Expression of the Hantaan virus G1 and G2 glycoproteins was accomplished by insertion of the **M** segment into the NYVAC and ALVAC vectors under the control of the entomopoxvirus 42 kDa promoter. The poxvirus expression cassette. . .

DETD A cDNA clone of the Hantaan virus **M** segment was derived as described by Schmaljohn et al. (1987) and provided by Dr. J. Dalrymple (Virology Division, U.S. Army. . . full sequence of the cDNA was presented previously by Schmaljohn et al. (1987). The 326 bp 5'-most region of the **M** segment coding sequence was derived using the plasmid pTZ19R containing the **M** segment cDNA as template and oligonucleotides HM5P (SEQ ID NO:335) (5'-ATGGGGA TATGGAAGTGG-3') and HM3P (SEQ ID NO:336) (5'-CATGTT CCTTTCAAGTCAAC-3'). This. . .

DETD The 3'-most 748 bp of the **M** segment coding sequence was derived by PCR using the cDNA clone contained in pTZ19R as template and oligonucleotides HMTS-5 (SEQ. . .

DETD The plasmid containing the **M**-specific cDNA clone in pTZ19R was used to transform GM48 (Dam^-) bacterial cells (BRL, Gaithersburg, Md.). Plasmid DNA derived from this. . . the 42 kDa promoter fused to the 5' most region of the coding sequence. The resultant plasmid containing the entire **M** segment expression cassette was designated as pBSHVM. The entire **M** segment cassette was excised from pBSHVM using restriction endonucleases HindIII and EcoRI. The 3508 bp derived fragment was blunt-end using the Klenow fragment of the **E**. coli in the presence of 2 mM dNTPs. The blunt ended fragment was inserted into pSD550 to yield pHVMVC.

DETD . . . vP882. Recombinant virus was identified by in situ hybridization according to standard procedures (Piccini et al., 1987) using a radiolabeled **M**-specific DNA probe. Recombinant plaques were purified by 3 rounds of plaque purification and amplified for further analysis. Recombinant virus, vP882, contains the Hantaan **M** segment in the 14L locus of vaccinia virus. Replacement of the I4L open reading frame with the **M** segment cassette in the vP804 background creates a NYVAC- equivalent virus background (Tartaglia et al., 1992).

DETD The 3508 bp HindIII/EcoRI fragment derived from PBSHVM, containing the **M** segment cassette (above), was inserted into pC4I digested with HindIII and EcoRI. The plasmid pC4I was derived as follows. A. . .

DETD Insertion of the **M** segment cassette into pC4I yielded plasmid pC4HVM. The plasmid pC4HVM was linearized with SmaI for insertion of a 100 bp. . . pC4HVMVQ was digested with SmaI followed by a subsequent partial HindIII digestion to recover a 3.6 kb fragment containing the **M** segment cassette. This fragment was blunt-ended using the klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This blunt-ended fragment was inserted into SmaI digested pSPCPC3L to. . .

DETD . . . identify and to purify the recombinant virus (as above; Piccini et al., 1987). The ALVAC-based recombinant containing the Hantaan virus **M** segment was designated as vCP114.

DETD . . . by linearization with XbaI followed by a partial HindIII digestion. This fragment was blunt-ended using the klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs and then inserted into the SmaI site of pSD541 (defined. . .

DETD . . . in the ATI site and vP951 contains this cassette at the same locus, but by virtue of rescue with the **M** segment containing vP882, also contains the **M** segment in the I4L locus.

DETD The plasmid PBSHVM was linearized with SalI and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This was ligated to the 1.4 kb XbaI/partial HindIII (Blunt-ended. . . from pBSHVS containing the Hantaan S segment expression cassette. The derived plasmid was designated as pBSHVMS. This plasmid contained

the M and S cassettes in a head to head configuration. Plasmid pBSHVMS was linearized with XhoI, blunted with Klenow (as above),. . .

DETD . . . a 1.5 kb fragment containing the S segment expression cassette. This fragment was blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs and inserted into the SmaI site of pSPCP3L (defined in. . .

DETD Expression Analysis of the NYVAC- and ALVAC-Based Hantaan Virus **M** and S Segment Recombinants

DETD . . . Schmaljohn (Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Md.). The recombinant viruses containing the **M** segment alone (vP882, and vCP114) or in combination with the S segment (vP951) displayed intense surface fluorescence using any of. . .

DETD . . . inoculated with NYVAC-based Hepatitis B virus (HBV) recombinants vP856, vP930, vP932 and vP975 (Example 13). vP856 expresses spsAg, the middle (**M**) form of the surface antigen. vP930 expresses lpsAg, the large (L) form of the surface antigen. vP932 expresses both spsAg. . .

DETD TABLE 34

AUSAB and CORAB
Rabbits
Analysis of sera of rabbits inoculated with NYVAC-based HBV recombinants expressing the
middle (**M**) form of the surface antigen (small pre S antigen), the large (L) form of the
surface antigen (large pre S antigen) and. . . 2 regions fused to the core antigen.

#	vP	HBV genes	week 1	2	3	4	5	6	7	8
AUSAB[a]										
A133	932	**M** + L	>512	>512	262	352	13000	6500	3600	5400
A134	932	**M** + L	250	235	72	80	3900	561	800	218
A135	975	**M** + L + S/C	36	58	274	406	1300	646	436	268
A136	975	**M** + L + S/C	103	>512	127	136	13468	4968	3168	2768

CORAB-

A135 975

M + L + S/C

80 20

20

80

320

80

320 80

A136 975

M + L + S/C

20 5

5 5 80

80

320 80

Rabbits were inoculated with 10^8 pfu of the. . .

DETD TABLE 35

Pre-S2 ELISA

Rabbits

Analysis by ELISA of sera from rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form

of the surface antigen and a fusion protein (S/C) consisting of the pre. . .

+ 2 regions

fused to the core antigen.

week

vP HBV genes

1 2 3 4 5 6 7 8

A133

932

M + L 0 0 29 35 474

602 358

419

A134

932

M + L 0 0 0 277 2017

3099

847

500

A135

975

M + L + S/C

0 0 0 0 175

105 94

48

A136

975

M + L + S/C

0 0 0 0 2440

763 672

355

Rabbits were inoculated by the intramuscular (IM) route. . .

DETD TABLE 36

Pre-S1 ELISA

Rabbits

Analysis by ELISA test of sera from rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form and the large (L) form of the surface antigen and the preS1 + 2/core fusion protein (S/C).

week

vP HBV genes 0 2 4 5 6 8

A133	932	**M** + L	<10	<10	<10	<10	<10	<10
A134	932	**M** + L	<10	<10	<10	<10	17	<10
A135	975	**M** + L + S/C						
			<10	<10	15	40	<10	24
A136	975	**M** + L + S/C						
			15	16	17	117	52	49

Rabbits were inoculated by the intradermal (ID) route with 10^8. . .

DETD TABLE 37

Pre-S2 ELISA

Guinea Pigs

Analysis by ELISA of sera from guinea pigs inoculated with NYVAC-based HBV recombinants expressing the middle (**M**) form of the surface antigen, the large (L) form of the surface antigen and the preS1 + 2/ core fusion protein (S/C).

#	vP	HBV genes	week 0	5	6
85	856	**M**	<10	<10	<10
86	856	**M**	<10	<10	<10
87	930	L	<10	46	35
88	930	L	<10	30	93
89	932	**M** + L	<10	39	<10
90	932	**M** + L	<10	33	19
91	975	**M** + L + S/C			
			<10	22	84
92	975	**M** + L + S/C			
			<10	53	269

Guinea pigs were inoculated by the SC route with 10^8 of the indicated
HBV recombinant vaccinia. . .

DETD TABLE 38

CORAB

Mice

Analysis of sera by CORAB test of mice inoculated with vaccinia recombinant vP975 expressing the HBV middle (**M**) form of the surface antigen, the large (L) form of the surface antigen and a fusion protein (S/C) consisting of the pre. . . 2 regions fused to the core antigen.

Group	vP	HBV genes	Week 1	2	3	4	5	6	7	8
D	975	**M** + L + S/C								
			--[a]							
				--	--	--	5	5	5	5

Mice were inoculated by the IM route with. . .

DETD TABLE 39

Pre-S2 ELISA

Mice

Analysis by ELISA of sera from mice inoculated with NYVAC-based HBV recombinants expressing the middle (**M**) form of the surface antigen, the large (L) form of the surface antigen and the preS1 + 2/core fusion protein (S/C).

	vP	HBV genes	week 0	5	6
Group A					
	856	**M**	<10	73	70
Group B					
	930	L	<10	93	112
Group C					

	932	M + L	<10	970	1140
Group D					
	975	M + L + S/C	<10	1054	1062

Groups of eight or twelve mice were inoculated by the IM route with. . .

DETD TABLE 40

Pre-S1 ELISA

Mice

Analysis by ELISA of sera from mice inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and the preS1 + 2/core fusion protein (S/C).

	vP	HBV genes	week 0	5
Group B	930	L	60	244
Group C	932	M + L	66	125
Group D	975	M + L + S/C	63	1554

Groups of eight or twelve mice were inoculated by the IM route with 10^7. . .

DETD . . . monolayers were either infected with parental virus, CPpp (ALVAC) or vPS66 (NYVAC), or infected with vCP1661 or vP1075 at an m.o.i. of 10 pfu/cell. Cells were infected, incubated in modified Eagle's medium (minus methionine) containing [^{35} S]-methionine (20 µCi/ml), lysed and. . .

DETD . . . by Makoff et al., 1989) for fragment C produced by papain digestion of native tetanus toxin as well as an E. coli produced recombinant fragment C which is identical to that encoded by vCP161 and vP1075.

DETD . . . days post-challenge. NYVAC-based pseudorabies virus recombinant viruses were all shown to reduce the effects of the virulent pseudorabies virus challenge (i.e. clinical signs and virus isolation) compared to the controls, with the gp50 expressing recombinant virus being the most efficacious. In. . .

DETD . . . 42 49

Animals receiving vP1015							
A168	<1.3[d]	<1.3	<1.3	1.3	2.2	2.2	2.2
A169	<1.3	1.6	1.6	1.6	3.1	3.1	2.5
Animals receiving vP913							
A116	<1.3	<1.3	N.D[e]	<1.3	2.8	2.2	2.2
A117	<1.3	<1.3	N.D.	<1.3	1.9	1.9	1.9

[a] Day of inoculation with 8.0 $\log_{10}$ pfu of. . .

DETD Construction of Insertion Vector Containing Japanese Encephalitis Virus (JEV) 15aaC, prM, E, NS21, NS2A

DETD . . . promoter, plasmid origin of replication and C5 flanking arms isolated. Plasmid JEVL14VC containing JEV cDNA encoding 15 amino acids C, prM, E, NS1, NS2A in a vaccinia virus donor plasmid (Mason et al., 1991) (nucleotides 337-4125, Konishi et al., 1991) was digested. .

DETD Construction of C5 Insertion Vector Containing JEV 15aaC, prM, E

DETD . . . annealed oligonucleotides SP131 (SEQ ID NO:382) and SP132 (SEQ ID NO:383) (containing a SphI sticky end, T nucleotide completing the E coding region, translation stop, a vaccinia early transcription termination signal (AT5AT; Yuen and Moss, 1987), a second translation stop, and XbaI sticky end) generating plasmid JEVCP5 which encodes 15 amino acids C, prM and E under the control of the H6 promoter between C5 flanking arms.

DETD JEVCP1 was transfected into ALVAC infected primary CEF cells to generate the canarypox recombinant vCP107 encoding 15 amino acids C, **prM**, **E**, NS1, NS2A. JEVCP5 was transfected into ALVAC infected primary CEF cells to generate the canarypox recombinant VCP140 encoding JEV 15 aa C, **prM** and **E**. ##STR41##

DETD Immunoprecipitation experiments were performed as described previously (Konishi et al., 1991). The **E** protein produced in vCP107 and vCP140 infected cells comigrates with the **E** protein produced by JEV-vaccinia recombinants which have been shown to produce an authentic **E** protein (Konishi et al., 1991). vCP107 produces an NS1 protein that comigrates with the NS1 protein produced by JEV-vaccinia recombinants. . .

DETD TABLE 48

Protective efficacy of TROVAC-NDV (vFP96) in SPF and commercial broiler chickens.

Bird Group	Dose	NDV HI GMT[d] Week 3	NDV HI GMT[d] Week 4	Percent Protection[e] NDV	Percent Protection[e] FP
Group 1[a]					
	2.0	<5	<5	70	100
	4.0	<5	<5	70	100
	None. . .	history of vaccination with fowlpox virus			

[c] : Specific pathogen free birds

[d] : Geometric mean titer of HI antibody

[e] : Percent protection of birds after NDV or Fowpox challenge

DETD . . . centrifugation and resuspended in Assay Medium (RPMI 1640 containing 10% fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, 5×10^{-5} **M** 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin). For memory CTL activity, the spleen cells from immunized mice were resuspended in Stimulation Medium (Minimum Essential Medium with Earle's salts containing 10%. fetal bovine serum, 2mM L-glutamine, 10^{-4} **M** 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin) and stimulated in vitro in upright 25 cm^2 tissue culture flasks with. . . titrated effector cells in 96-well microtiter plates for a 4 hr ^{51} Cr release assay. Effector to target cell ratios (**E**:T) shown for the three assays were 100:1 (primary), 20:1 (memory), and 50:1 (secondary). Percent cytotoxicity was calculated as (experimental ^{51}. . .

DETD . . . and resuspended in the original volume of Cytotoxicity Medium, divided into two equal portions with or without complement (Rabbit Lo-Tox **M**, Cedarlane) and incubated at 37° C. for 45 min. The cells were then washed in Assay Medium and, based on. . .

DETD . . . apparent molecular masses of 160 kDa, 120 kDa, and 41 kDa, respectively. These are consistent with expression of the precursor **envelope** glycoprotein (160 kDa) and the proteolytically derived mature forms (120 kDa and 41 kDa).

DETD . . . digested pC5L to yield pC5HIV3BEEC. A 2.7 kb NruI/XbaI fragment from pBSHIV3BEECM was blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase and inserted into NruI/SmaI digested pSPHAH6 to yield pHAHIV3BEEC.

DETD . . . by isolating the 2.1 kb NruI/XbaI fragment from pBSHIVMNT. This fragment was then blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs and inserted into pSPHAH6 digested with NruI and SmaI to. . .

DETD . . . Corp., Emeryville, Calif.). Investigation of surface immunofluorescence indicated that vCP138 and vP1035 infected cells contained HIV-1(MN) gp120 in the plasma **membrane**. Significantly, the surface staining of vCP138 and vP1035 infected cells was greatly enhanced compared to cells infected with recombinant viruses (i.**e**. vCP125, vCP124, vP1004, and vP1008) expressing gp160 or a non-anchored gp120. Results from immunoprecipitation analyses confirmed the expression of gp120. . .

DETD . . . precursor protein. Vero cell monolayers were either mock infected, infected with the parental virus or infected with vP969 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD An H6-promoted truncated HIV-1 **envelope** gene was then inserted into pHIVG4. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10, containing an H6-promoted truncated HIV-1 **envelope** gene, into the filled-in BamHI site of pHIVG4. The plasmid generated by this manipulation is called pHIVGE11.

DETD . . . New Haven, Conn.). The plasmid pBSHIV3BCDT1 contains an H6 promoted cassette to express a severely truncated form of the HIV-1(IIIB) **envelope** (amino acid 1 to 447; Ratner et al., 1985). Expression of this cassette was evaluated to eliminate CD4 binding while. . .

DETD An H6-promoted truncated HIV-1 **envelope** gene was then inserted into pHIVG7. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10 (defined in Example 95), containing an H6-promoted truncated HIV-1 **envelope** gene, into the filled-in BamHI site of pHIVG7. The plasmid generated by this manipulation is called pHIVGE12.

DETD The H6-promoted HIV-1(MN) **envelope** (gp120) gene was then inserted into pHIVGE14. This was accomplished by cloning the oligonucleotides, HIVL29 (SEQ ID NO:421) (5'-GGCCGCAAC-3') and. . .

DETD The H6-promoted **envelope** (gp120) gene and the I3L-promoted gag and pol genes were then inserted into a vaccinia virus insertion vector. This was. . .

DETD . . . by the gp160 gene. This was accomplished by cloning the 2,600 bp NruI-NotI fragment of pH6HMNE, containing the entire HIV-1(MN) **envelope** (gp160) gene, into the 8,000 bp partial NruI-NotI fragment of pHIVGE16. The plasmid generated by this manipulation is called pHIVGE19.

DETD . . . gene products. CEF cell monolayers were either mock infected, infected with the parental virus or infected with vCP117 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . by the gp160 gene. This was accomplished by cloning the 2,600 bp NruI-NotI fragment of pH6HMNE, containing the entire HIV-1(MN) **envelope** (gp160) gene, into the 9,800 bp NruI-NotI fragment of pHIVGE15. The plasmid generated by this manipulation is called pHIVGE18.

DETD . . . gene products. CEF cell monolayers were either mock infected, infected with the parental virus or infected with VCP130 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gag-pol and env genes would also produce such particles. Furthermore, if these ALVAC-based recombinants were used to infect non-avian cells (i.**e**. Vero, MRC-5, etc.) then HIV-1 virus-like particles could be purified without any poxvirus virion contaminants.

DETD . . . evaluate particle formation using Vero cells infected with vCP156, the following experiment was performed. Vero cells were infected at an **m**.o.i. of approximately 5 pfu/cell. After a 24 hr infection period, the supernatant was harvested and clarified by centrifugation at 2000. . . With the size exclusion noted above, the p24 would have passed through unless it was in a higher structural configuration (i.**e**. virus-like particles). Therefore, these results strongly suggest that HIV-1 virus-like particles containing the gp120 **envelope** component are produced in vCP156 infected cells.

DETD . . . gene products. Vero cell monolayers were either mock infected, infected with the parental virus or infected with vP1045 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gene products. CEF cell monolayers were either mock infected, infected with the parental virus or infected with vCP153 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . precursor proteins. Vero cell monolayers were either mock infected, infected with the parental virus or infected with vP948 at an

m.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD Macaque sera from SIV seropositive individuals specifically precipitated the SIV gag precursor protein and the **envelope** glycoprotein from vP948 infected cells, but did not precipitate SIV-specific proteins from mock infected cells.

DETD The plasmid, pSIVEMVC, contains the H6-promoted SIV_{MAC142} **envelope** gene (in vitro selected truncated version). The region of the **envelope** gene containing the premature termination codon was cloned into pBSK+. This was accomplished by cloning the 1,120 bp ClaI-BamHI fragment. . .

DETD At day 56 (i.**e**. 28 days after the second injection) protective titers were achieved in 0/3 of Group A, 2/3 of Group B and. . .

DETD . . . 35 56

1	$10^3.5$					
		<0.1	<0.1	<0.1	<0.1	0.2
3	$10^3.5$					
		<0.1	<0.1	<0.1	<0.1	<0.1
4	$10^3.5$					
		<0.1	<0.1	<0.1	<0.1	<0.1
	G.**M**.T.	<0.1	<0.1	<0.1	<0.1	<0.1
6	$10^4.5$					
		<0.1	<0.1	<0.1	<0.1	<0.1
7	$10^4.5$					
		<0.1	<0.1	<0.1	2.4	1.9
10	$10^4.5$					
		<0.1	<0.1	<0.1	1.6	1.1
	G.**M**.T.	<0.1	<0.1	0.1	0.58	0.47
11	$10^5.5$					
		<0.1	<0.1	1.0	3.2	4.3
13	$10^5.5$					
		<0.1	<0.1	0.3	6.0	8.8
14	$10^5.5$					
		<0.1	<0.1.	. .	0.3	3.7
21	$10^5.5$					
		<0.1	<0.1	0.2	2.6	3.9
23	$10^5.5$					
		<0.1	<0.1	<0.1	1.7	4.2
25	$10^5.5$					
		<0.1	<0.1	<0.1	0.6	0.9
	G.**M**.T.	<0.1	<0.1	0.16	1.9	4.4*
2	HDC	<0.1	<0.1	0.8	7.1	7.2
5	HDC	<0.1	<0.1	9.9	12.8	18.7
8	HDC	<0.1	<0.1.	. .	7.7	20.7
19	HDC	<0.1	<0.1	2.6	9.9	9.1
22	HDC	<0.1	<0.1	1.4	8.6	6.6
24	HDC	<0.1	<0.1	0.8	5.8	4.7
	G.**M**.T.	<0.1	<0.1	2.96	9.0	11.5*

* p = 0.007 student t test

DETD PROTECTION AGAINST JAPANESE **ENCEPHALITIS VIRUS** BY NYVAC-JEV RECOMBINANTS (vP908, vP923)

DETD Using NYVAC-JEV recombinants, protection against Japanese **Encephalitis virus** was provided. NYVAC vP866, NYVAC recombinants vP908 and vP923, and vaccinia recombinants vP555 and vP829 were produced as described herein.

DETD . . . positioned behind the early/late H6 promoter. Recombinant vP908 (and vP555; Mason et al., 1991) includes the putative 15 amino acid **signal sequence** preceding the N-terminus of **prM**, **prM**, **E**, NS1 and NS2A. Recombinant vP923 (and vP828; Konishi et al., 1991) encodes the putative **signal sequence** of **prM**, **prM**, and **E**.

DETD Synthesis of **E** and NS1 by Recombinant Vaccinia Viruses. Immunoprecipitation of the **E** or NS1 gene was performed using a monoclonal antibody specific for **E** or NS1. Proteins reactive with the

E MAb were synthesized in cells infected with vP555, vP908 and vP923, and proteins reactive with NS1 MAb were synthesized in. . . cells infected with vP555 and vP908 but not in cells infected with vP923. vP555 infected cells produced correct forms of **E** and NS1 inside and outside of the cell. The proteins produced by vP908 and vP923 were identical in size to those produced by vP555. For both **E** and NS1, the extracellular forms migrated slower than the intracellular forms in SDS-PAGE, consistent with maturation of the N-linked glycans. . . the JEV genome (Mason et al., 1987). Immunoprecipitates prepared from radiolabeled vaccinia recombinant infected cells using a MAb specific for **M** (and **prM**) revealed that **prM** was synthesized in cells infected with vP908 and vP923.

DETD The immune response to **E** correlated well with the results of the NEUT and HAI tests. The RIP response to **E** observed in swine immunized with vP923 on day 35 was higher than the RIP response to **E** in swine immunized with vP908, whereas the HAI titers on day 35 were equivalent in the two groups. However, NEUT. . . be induced but the quantitative aspects of the RIP analysis was not further validated. Weak RIP responses of sera to **E** on day seven in spite of relatively high NEUT antibody titers could be explained by IgM antibody early after immunization.. . .

DETD . . . sera collected 20 days post-challenge for antibodies against JEV. The swine vaccinated with vP908 or vP923 had higher responses to **E** than those inoculated with PBS or vP866, indicating that the antibody reactivity to **E** that was present before challenge was boosted by JEV infection. Reactions to NS3 and NS5, JEV proteins which were not. . .

DETD TABLE 52

Immunization and JEV challenge in mice

Immunizing Virus[a]	JEV Genes Expressed	Antibody titer NEUT[b]	HAI[c]	Survival[d]
vP829	**prM, E**	1:320	1:80	10/10 (100%)
vP866	None	<1:10	<1:10	0/12 (0%)
vP908	**prM, E,** NS	1:320	1:80	11/12 (92%)
vP923	**prM, E**	1:320	1:80	12/12 (100%)

[a] Vaccinia recombinant virus used for immunizing groups of 4week old mice.

[b] Serum dilution yielding. . .

DETD . . . recombinants have also been shown to elicit measles virus neutralizing antibodies in rabbits and protection against pseudorabies virus and Japanese **encephalitis virus** challenge in swine. The highly attenuated NYVAC strain confers safety advantages with human and veterinary applications (Tartaglia et al., 1990).. . .

DETD . . . skin); c) absence of testicular inflammation (nude mice); d) greatly reduced virulence (intracranial challenge, both three-week old and newborn mice); **e**) greatly reduced pathogenicity and failure to disseminate in immunodeficient subjects (nude and cyclophosphamide treated mice); and f) dramatically reduced ability. . .

DETD 1. Adamowicz, Ph., F. Tron, R. Vinas, **M**. N. Mevelec, I. Diaz, A. **M**. Courouce, **M**. C. Mazert, D. Lagarde and **M**. Girard, In Viral Hepatitis and Liver Disease, pp. 1087-1090 (1988).

DETD . . . Alexander, D. J. In Diseases of Poultry, 9th edition, eds. B. W. Calnek, H. J. Barnes, C. W. Beard, W. **M**. Reid and H. W. Yoder, Jr., (Iowa State University Press, Ames, Iowa, USA) pp. 496-519 (1991).

DETD 8. Arikawa, J., Schmaljohn, A. L., Dalrymple, J. **M**., and Schmaljohn, C. S., J. Gen. Virology 70, 615-624 (1989).

DETD 9. Asada, H., Tamura, **M**., Kondo, K., Dohi, Y, Yamanishi, K., J. Gen Virology 69, 2179-2188 (1988).

DETD 10. Asada, H. M., Yamura, K., Kondo, T., Ohno, T., Takahashi, T., Dohi, T., Nagai, T., Kurata, T., and Yamanishi, K., J. Gen. Virology. .

DETD 12. Baer. R., Bankie, A. T., Biggin. M. D., Deiniger. P. L., Farrel. P. J., Gibson. T. J., Hatfull. G., Hudsson. G. S., Satchwell. S. C., Seguin. C.,. . .

DETD 15. Baxby D., Paoletti E., Vaccine 9, 8-9 (1992).

DETD 17. Beard, C. W., and R. P. Hanson, In Disease of poultry, 8th edition, ed. M. S. Hofstad, (Iowa State University Press, Ames, Iowa) pp. 452-470 (1984).

DETD 18. Beattie, E., Tartaglia, J., and Paoletti, E., Virology 183, 419-422 (1991).

DETD 19. Behbehani, A. M., Microbiological Reviews 47, 455-509 (1983).

DETD 23. Ben-Porat, T., F. J. Rixon, and M. L. Blankenship, Virology 95, 285-294 (1979).

DETD 25. Bergoin, M., and Dales, S., In Comparative Virology, eds. K. Maramorosch and E. Kurstak, (Academic Press, NY) pp. 169-205 (1971).

DETD 26. Berns, K. I., In: Fields Virology, eds. B. N. Fields and D. M. Knipe, (Raven Press, New York) pp. 1743-1763 (1990).

DETD 29. Borysiewicz, L. K., J. K. Hickling, S. Graham, J. Sinclair, M. P. Crange, G. L. Smith, and J. G. Sissons, J. Exp. Med. 168, 919-931 (1988).

DETD 30. Boursnell, M. E. G., P. F. Green, J. I. A. Campbell, A. Deuter, R. W. Peters., F. M. Tomley, A. C. R. Samson, P. Chambers, P. T. Emmerson, and M. M. Binns, J. Gen. Virol. 71, 621-628 (1990a).

DETD 31. Boursnell, M. E. G., P. F. Green, J. I. A. Campbell, A. Deuter, R. W. Peters, F. M. Tomley, A. C. R. Samson, P. T. Emmerson, and M. M. Binns, Veterinary Microbiology 23, 305-316 (1990b).

DETD 32. Boursnell, M. E. G., P. F. Green, A. C. R. Samson, J. I. A. Campbell, A. Deuter, R. W. Peters, N. S. Millar, P. T. Emmerson, and M. M. Binns, Virology 178, 297-300. (1990c).

DETD 33. Boursnell, M. E. G., I. J. Foulds, J. I. Campbell and M. M. Binns, J. Gen. Virol. 69, 2995-3003 (1988).

DETD 34. Brandt, W. E., J Infect Dis. 157, 1105-1111 (1988).

DETD 35. Brochier B., Kieny M. P., Costy F., et al., Nature, 354 520-522 (1991).

DETD 36. Bryson, Y., Dillon, M., Lovett, M., Acuna, G., Taylor, S., Cherry, J., Johnson, B., Wiesmeier, E., Growdon, W., Creagh-Kirk, T. and Keeney , R., N. Engl. J. Med. 308, 916-921 (1983).

DETD 37. Bucher, D., Popplo, S., Baer, M., Mikhail, A., Gong, Y-F., Whitaker, C., Paoletti, E., and Judd, A., J. Virol. 63, 3622-3633 (1989).

DETD 38. Buller, R. M. L., and Palumbo, G. J., Microbiol. Rev. 55, 80-122 (1991).

DETD 39. Buller, R. M. L., Chakrabarti, S., Cooper, J. A., Twardzik, D. R., and Moss, B., J.Virol. 62, 866-874 (1988).

DETD 41. Bunn, T. O., In: Rabies, eds. Campbell, J. B. and Charlton K. M. (Kluwer Academy Press, Boston) pp. 474-491 (1988).

DETD 42. Cantin, E., Eberle, R., Baldrick, J., Moss, B., Willey, D., Notkins, A. and Openshaw, H., Proc. Natl. Acad. Sci. USA, 84, 5908-5912. . .

DETD 44. Chakrabarti, S., Robert-Guroff, M., Wong-Staal, F., Gallo, R. C., and Moss, B. Nature 320, 535-537 (1986)

DETD 45. Chambers, T. M., Y. Kawaoka, and R. G. Webster, Virology 167, 414-421 (1988).

DETD 49. Charles, I. G., Rodgers, B. C., Nakoff, A. J., Chatfield, S. N., Slater, D. E., and Fairweather, N. F., Infect. Immun. 59, 1627-1632 (1991).

DETD 52. Child, S. J., Palumbo, G. J., Buller, R. M. L., and Hruby, D. E. Virology 174, 625-629 (1990).

DETD 53. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J., Biochemistry 18, 5294-5299 (1979).

DETD 54. Chisari, F. V., P. Filippi, A. McLachlan, D. R. Milich, M. Riggs, S. Lee, R. R. Palmiter, C. A. Pinkert and R. L. Brinster, J. Virol. 60, 880-887 (1986).

DETD 56. Clark, N., Kushner, B. S., Barrett, M. S., Kensil, C. R.,

Salisbury, D., and Cotter, S., JAMA 199, 1439-1442 (1991).
DETD 57. Clarke, B. **E**., S. **E**. Newton, A. R. Carroll, N. J. Francis, G. Appleyard, A. D. Syred, P. **E**. Highfield, D. J. Rowlands and F. Brown, Nature 330, 381-384 (1987).
DETD 61. Colinas, R. J., R. C. Condit and **E**. Paoletti, Virus Research 18, 49-70 (1990).
DETD 62. Collett, **M**. S., Keegan, K., Hu, S.-L, Sridhar, P., Purchio, A. F., Ennis, W. H., and Dalrymple, J. **M**., In: The Biology of Negative Strand Viruses, pp. 321-329 (1987).
DETD 64. Cooney **E**. L., Corrier A. C., Greenberg P. D., et al., Lancet 337, 567-572 (1991).
DETD 68. Dantas, J. R., Fr., Okuno, Y., Asada, H., Tamura, **M**., Takahashi, **M**., Tanishita, O., Takahashi, Y. Kurata, T., and Yamanishi, K., Virology 151, 379-384 (1986).
DETD 70. De, B. K., **M**. W. Shaw, P. A. Rota, **M**. W. Harmon, J. J. Esposito, R. Rott, N. J. Cox and A. P. Kendal, Vaccine 6, 257-261 (1988).
DETD 71. Delpeyroux, F., N. Peillon, B. Blondel, R. Crainic and R. **E**. Streeck, J. Virol., 62, 1836-1839 (1988).
DETD 72. DeNoronha, F., Schafer, W., and Essex, **M**., Virology 85, 617-621 (1978).
DETD 73. Derosiers, R. C., **M**. S. Wyand, T. Kodama, T. J. Ringler, L. O. Arthur, P. K. Sehgal, N. L. Letvin, N. W. King and **M**. D. Daniel, Proc. Natl. Acad. Sci. USA 86, 6353-6357 (1989).
DETD 75. Douglas, J., Critchlow, C., Benedetti, J., Mertz, G., Connor, J., Hintz, **M**., Fahnlander, A., Remington, **M**., Winter, C. and Corey, L., N. Engl. J. Med. 310, 1551-1556 (1984).
DETD 77. Dowling, P. C., B. **M**. Blumberg, J. Menonna, J. **E**. Adamus, P. Cook, J. C. Crowley, D. Kolakofsky, and S. D. Cook, J. Gen. Virol. 67, 1987-1992 (1986).
DETD 82. Eble, B. **E**., V. R. Lingappa and D. Ganem, Mol. Cell. Biol. 6, 1454-1463 (1986).
DETD 83. Edbauer, C., R. Weinberg,. J. Taylor, A. Rey-Senelonge, J. F. Bouquet, P. Desmettre, and **E**. Paoletti, Virology 179, 901-904 (1990).
DETD 84. Eisel, U., Jarausch, W., Goretzki, K., Henschen, A., Engels, J., Weller, U., Hudel, **M**., Habermann, **E**., and Niemann, H. EMBO J. 5, 2495-2502 (1986).
DETD 85. Elder, J. H., McGee, J. S., Munson, **M**., Houghton, R. A., Kloetzer, W., Bittle, J. L., and Grant, C. K., J. Virol. 61, 8-15 (1987).
DETD 88. Espion, D., S. de Henau, C. Letellier, C.-D. Wemers, R. Brasseur, J. F. Young, **M**. Gross, **M**. Rosenberg, G. Meulemans and A. Burny. Arch. Virol. 95, 79-95 (1987).
DETD 91. Etinger H. **M**., Altenburger W., Vaccine 1991, 9, 470-472.
DETD 98. Franchini, G., Gurgo, C., Guo, H.-G., Gallo, R. C., Collati, **E**., Fargnoli, K. A., Hall, L. F., Wong-Staal, F., and Reitz, Jr., **M**. S., Nature (London) 328, 539-543 (1987).
DETD 99. Franchini, G., Fargnoli, K. A., Giomnini, F., Jagodzinski, L., DeRossi, A., Bosch, **M**., Biberfield, G., Fenyo, **E**. **M**., Albert, J., Gallo, R. C., and WongStaal, F., Proc. Natl. Acad. Sci. USA 86, 2433-2437 (1989).
DETD 102. Galibert, F., **E**. Mandart, F, Fitoussi, P. Tiollais and P. Charnay, Nature 281, 646-650 (1979).
DETD 109. Goebel S. J., Johnson G. P., Perkus **M**. **E**., Davis S. W., Winslow J. P. and **E**. Paoletti, Virology 179, 247-266 (1990).
DETD 110. Goebel, S. J., G. P. Johnson, **M**. **E**. Perkus, S. W. Davis, J. P. Winslow and **E**. Paoletti, Virology 179, 517-563 (1990b).
DETD 112. Gonczol, **E**., Furlini, G., Ianacone, J, and Plotkin, S. A., J. Virol. 14, 37-41 (1986).
DETD 113. Gonczol, **E**., C. de Taisne, G. Hirka, K. Berensci, W. Lin, **E**. Paoletti, and S. Plotkin, Vaccine 9, 631-637 (1991).
DETD 114. Gonzolez-Scarano, F., Shope, R. **E**., Calisher, C. H., and Nathanson, N, Virology, 120, 42-53 (1982).
DETD 115. Gould, **E**. A., Buckley, A., Barrett, A. D. T., and Cammack, N., J. Gen. Virol. 67, 591-595 (1986).
DETD 116. Graves, **M**. C., J. **M**. Silver, and P. W. Choppin, Virology 86, 254-263 (1978).
DETD 117. Gretch, D. R., B. Kari, L. Rasmussen, R. C. Gehrz, and **M**. F.

Schnski, S. Virol. 62, 875-881 (1988).
DETD 120. Guo, P., Goebel, S., Perkus, M. E., Taylor, J., Norton, E., Allen, G., Languet, B., Desmettre, P., and Paoletti, E., J. Virol. 64, 2399-2406 (1990).
DETD 121. Guo, P., Goebel, S., Davis, S., Perkus, M. E., Languet, B., Desmettre, P., Allen, G., and Paoletti, E., J. Virol. 63, 4189-4198 (1989).
DETD 122. Guo, H-G., diMarzo Veronese, F., Tschachler, E., Pal, R., Kalyanaraman, V. S., Gallo, R. C., and Reitz, Jr., M. S., Virology 174, 217-224 (1990).
DETD 124. Gurgo, C., Guo, H.-G., Franchini, G., Aldovini, A., Collalti, E., Farrell, K., Wong-Staal, F., Gallo, R. C., and Reitz, M. S., Jr., Virology 164, 531 (1988).
DETD 126. Halpern, J. L., Habig, W. H., Neale, E. A., and Stibitz, S. Infect. Immun. 58, 1004-1009 (1990).
DETD 128. Hardy, Jr., W. D., Hess, P. W., MacEven, E. G., McClelland, A. J., Zuckerman, E. E., Essex, M., Cotter, S. M., and Jarrett, O., Cancer Res. 36 582-588 (1976).
DETD 130. Hashimura K., Kaminiyada M., Akazaki M., Yonemaru K., Okuzono Y., Hukomoto M., Miura Y., and Hayashi S., J. Vet. Med. Sci. 34, 314-319 (in Japanese with English summary) (1981).
DETD 132. Henchal, E. A., Henchal, L. S., and Schlesinger, J. J., J. Gen. Virol. 69, 2101-2107 (1988).
DETD 136. Homma, M., and M. Ohuchi, J. Virol. 12, 1457-1465 (1973).
DETD 137. Hoshikawa, N., Kojima, A., Yasuda, A., Takayashiki, E., Masuko, S., Chiba, J., Sata, T., and Kurata, T., J. Gen. Virol. 72, 2509-2517 (1991).
DETD 138. Hosmalin, A., Nara, P. L., Zweig, M., Lerche, N. W., Cease, K. B., Gard, E. A., Markham, P. D., Putney, S. D., Daniel, M. D., Desrosiers, R. C., and Berzofsky, J. A. J. Immunol. 146, 1667-1673 (1991).
DETD 139. Hruby, D. E., R. A. Maki, D. B. Miller and L. A. Ball, Proc. Natl. Acad. Sci. USA 80, 3411-3415 (1983).
DETD 140. Hruby, D. E. and L. A. Ball, J. Virol. 43, 403-409 (1982).
DETD 142. Hu, S.-L. Travis, B. M., Garrigues, J., zarling, J. M., Sridhar, P., Dykers, T., Eichberg, J. W., and Alpers, C. Virology 179, 321-329 (1990).
DETD 143. Hu, S.-L., Fultz, P., McClure, H., Eichberg, J., Thomas, E., Zarling, J., Singhal, M., Kosowski, S., Swenson, R., Anderson, D. and Todaro, G.., Nature 328, 721-723 (1987).
DETD 144. Hu S. L., Kosowski S. G., Dallyrmple J. M., Nature 320, 537-540 (1986).
DETD 145. Hu, S.-L., Travis, B. M., Garrigues, J., Zarling, J. M., Eichberg, J. W. and Alpers, C. E., In Vaccine 90, eds. Chanock, R. M., Lerner, R. A., Brown, F., and Ginsberg, H., (Cold Spring Harbor Press, Cold Spring Harbor, New York) pp. 231-236 (1990).
DETD 152. Ito H., Maruyama N., Maruyama S., Yamashita T., Tserumizu T., Ogonuki M., Matsui S., Fuse Y., Araki H., Nishi Y., Yamashita M., Takabata S., Sakurai Y., and Otake S., J. Vet. Med. Sci., 27, 331-334 (in Japanese with English summary) (1974).
DETD 153. Itoh, Y., E. Takai, H. Ohnuma, K. Kitajima, F. Tsuda, A. Machida, S. Mishiro, T. Nakamura, Y. Miyakawa and M. Mayumi, Proc. Natl. Acad. Sci. USA 83, 9174-9178 (1986).
DETD . . . Jacobson, J. G., D. A. Leib, D. J. Goldstein, C. L. Bogard, P. A. Schaffer, S. K. Weller and D. M. Coen, Virology 173, 276-283 (1989).
DETD 158. Jarrett, O., Hardy, Jr., W. D., Golder, M. C., and Hay, D., Int. J. Cancer 21, 334-337 (1978).
DETD 159. Jarrett, O., Laird, H. M., and Hay, D., J. Gen. Virol. 20, 169-175 (1973).
DETD 162. Jin, H. and Elliot, R. M., J. Virology 65, 4182-4189 (1991).
DETD 163. Joklik, W. K., Pickup, D. J., Patel, D. D., and Moody, M. D., Vaccine 6, 123-128 (1988).
DETD 164. Karacostas, V., Nagashima, K., Gonda, M. A., and Moss, B., Proc. Natl. Adad. Sci, USA 86, 8964-8968 (1989).
DETD 165. Kari, B., N. Lussenhop, R. Goertz, M. Wabuke-Bunoti, R. Radeke, and R. Gehrz, J. Virol. 60, 345-352 (1986).

DETD 166. Kato, S., M. Takahashi, S. Kameyama and O. Kamahora, Biken J 2, 353-363 (1959).
DETD 167. Kaufman, B. M., Summers, P. L., Dubois, D. R., and Eckels, K. H., Am. J Trop. Med. Hyg. 36, 427-434 (1987).
DETD 168. Kaufman, B. M., Summers, P. L., Dubois, D. R., Cohen, W. H., Gentry, M. I., Timchak, R. L., Burke, D. S. and Eckels, K. H., Am. J. Trop. Med. Hyg. 41, 576-580 (1989).
DETD 170. Keegan, K. and Collett, M. S., J. Virology 58, 263-270 (1986).
DETD 171. Kensil, C. R., Barrett, M. S., Kushner, B. S., Beltz, G., Storey, J., Patel, U., Recchia, J., Aubert, A., and Marciaini, D., JAVMA 199, 1402-1405. . .
DETD 172. Kieff, E., and Liebowitz, D., In Virology, Second Edition, eds. B. N. Fields, D. M. Knipe et al., (Raven Press) (1990).
DETD 173. Kieny, M. P., Lathe, R., Drillien, R., Spehner, D., Skory, S., Schmitt, D., Wiktor, T., Koprowski, H., and Lecocq, J. P., Nature. . .
DETD 174. Killington, R. A., J. Yeo, R. W. Honess, D. H. Watson, B. E. Duncan, I. W. Halliburton, and J. Muimford, J. gen. Virol. 37, 297-310 (1977).
DETD 176. Kingsbury, D. W., In Virology, Second Edition, eds. B. N. Fields, D. M. Knipe et al., (Raven Press, Ltd. New York) pp. 1075-1089 (1990).
DETD 177. Kingsbury, D. W., M. A. Bratt, P. W. Choppin, R. P. Hanson, T. Hosaka, Y. ter Meulen, E. Norrby, W. Plowright, R. Rott and W. H. Wunner, Intervirology 10, 137-152 (1978).
DETD 181. Knauf, V. C., and Nester, E. W., Plasmid 8, 45-54 (1982).
DETD 183. Kodama, T., Wooley, D. P., Naidu, Y. M., Kestler III, H. W., Daniel, M. D., Li, Y. and Derosiers, R. C. J. Virol. 63, 4709-4714 (1989).
DETD 185. Konishi, E., Pincus, S., Fonseca, B. A. L., Shope, R. E., Paoletti, E., and Mason, P. W., Virology 185, 401-410 (1991).
DETD 187. Kost, T. A., E. V. Jones, K. M. Smith, A. P Reed, A. L. Brown, and T. J. Miller, Virology 171, 365-376 (1989).
DETD 191. Kotwal, G. J., S. N. Isaacs, R. McKenzie, M. M. Frank and B. Moss, Science 250, 827-830 (1990).
DETD 199. Kuwert E. K., Barsenbach C., Werner J., et al., In Cell Culture Rabies Vaccines and Their Protection Effect in Man, eds. Kuwert/Wiktor/Koprowski. . .
DETD 202. Lane, J. M., Ruben, F. L., Neff, J. M., and Millar, J. D., New Eng. J. Med. 281, 1201-1208 (1969).
DETD 203. Lathe, R., M. P. Kieny, D. Schmitt, P. Curtis and J. P. Lecocq, J. Mol. Appl. Gen. 2, 331-342 (1984).
DETD 205. Lecocq, J. P., M. P. Kieny, Y. Lemoine, R. Drillien, T. Wiktor, H. Koprowski and R. Lathe, In World's Debt to Pasteur, eds. Koprowski, . . .
DETD 206. Lecocq, J. P., M. Zukowski and R. Lathe, In Methods in Virology, eds. Maramorosch, K. and Koprowski, H., (Academic Press, New York) Vol. VII, . . .
DETD 208. Liu, Y-N.C., A. Klaus, B. Kari, M. F. Stinski, J. Exhkardt, and R. C. Gehrz, J. Virol. 65, 1644-1648 (1991).
DETD 212. Mackett M., Smith G. L., Moss B., Proc. Natl. Acad. Sci. 79, 7415-7419 (1982).
DETD 213. Makoff, A. J., Ballantine, S. P., Smallwood, A. E., and Fairweather, N. F. Bio/Technology 7, 1043-1046 (1989).
DETD 215. Maniatis, T., Fritsch, E. F., and Sambrook, J. Zn Molecular cloning: a laboratory manual, (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) (1982).
DETD 217. Marsden, H., Stow, N., Preston, V., Timbury, M. and Wilkie, N., J. Virol. 28, 624-642 (1978).
DETD 220. Mason P. W., Dalrymple J. M., Gentry M. K., McCown J. M., Hoke C. H., Burke D. S., Fournier M. J. and Mason T. L., J. Gen. Virol. 70, 2037-2049 (1989).
DETD 221. Mason, P. W., Pincus, S., Fournier, M. J., Mason, T. L., Shope, R. E., and Paoletti, E., Virol. 180, 294-305 (1991).
DETD 222. Mason, P. W., McAda, P. C., Dalrymple, J. M., Fournier, M. J., and Mason, T. L., Virology 158, 361-372 (1987a).
DETD 223. P. W., McAda, P. W., Mason, T. L., and Fournier, M. J., Virol. 161, 262-267 (1987b).

DETD 224. Mathes, L. E., Olsen, R. D., Hebebrand, L. C., Hoover, E. A., and Schaller, J. P., Nature 274, 687-691 (1978).
DETD 225. Matthews, R. E. F., Intervirology 17, 104-105 (1982a).
DETD 226. Matthews, R. E. F., Intervirology 17, 42-44 (1982b).
DETD 227. Mazzara, G. P, Destree, A. T., Williams, H. W., Sue. J. M., Belanger, L. M. and Panicali, D., Vaccines 87, 419-424 (1987).
DETD 228. McAda, P. C., Mason, P. W., Schmaljohn, C. S., Dalrymple, J. M., Mason, T. L. and Fournier, M. J. Virology 158, 348-360 (1987).
DETD 230. McLachlan, A., D. R. Milich, A. K. Raney, M. G. Riggs, J. L. Hughes, J. Sorge and F. V. Chisari, J. Virol. 61, 683-692 (1987).
DETD 231. McLaughlin-Taylor, E., Willey, D., Cantin, E., Eberle, R., Moss, B. and Openshaw H., J. Gen. Virol. 69, 1731-1734 (1988).
DETD 232. McGeoch, D., Moss, H., McNab, D. and Frame, M., J. Gen. Virol. 68, 19-38 (1987).
DETD 238. Meulemans, G., C. Letellier, M. Gonze, M. C. Carlier, and A. Burny, Avian Pathol. 17, 821-827 (1988).
DETD 239. Michel, F., Hoffenbach, A., Langlade-Demoyen, P., Guy, B., Lecocq, J.-P., Wain-Hobson, S., Kieny, M.-P. and Plata, F., Eur. J. Immunology 18, 1917 (1988).
DETD 243. Milich, D. R., G. B. Thornton, A. R. Neurath, S. B. Kent, M-L. Michel, P. Tiollais and F. V. Chisari, Science 228, 1195-1199 (1985).
DETD 245. Miller, G., In Virology, Second Edition, eds. B. N. Fields, D. M. Knipe et al., (Raven Press) (1990).
DETD 246. Monath, T. P., In The Togaviridae and **Flaviviridae**, eds. S. Schlesinger and M. J. Schlesinger, (Plenum Press, New York/London) pp. 375-440 (1986).
DETD 247. Morgan, A. J., M. Mackett, S. Finerty, J. R. Arrand, F. T. Scullion and M. A. Epstein, J. Med. Virol. 25, 189-195 (1988).
DETD 248. Morgan, J. R. and B. E. Roberts, J. Virol. 51, 283-297 (1984).
DETD 250. Moss, B., E. Winters and J. A. Cooper, J. Virol. 40, 387-395 (1981).
DETD 251. Mullins, J. I., and Hoover, E. A., In: Retrovirus Biology and Human Disease, (eds. Gallo, R. C., Wong-Staal, F.) Marcel Dekker, Inc., New York, pp. 87-116. . .
DETD 252. Murphy, B. R., and R. G. Webster, In: Virology, eds. Fields, B. N, Knope, D. M. et al., Raven Press, NY, 1091-1151 (1990).
DETD 253. Murphy-Corb, M., Martin, L. N., Davison-Fairburn, B., Montelaro, R. C., Miller, M., West, M., Ohkawa, S., Baskin, G. B., Zhang, J.-Y., Putney, S. D., Allison, A. C. and Eppstein, D. A., Science 246, 1293-1297. . .
DETD 254. Murray, K., S. A. Bruce, A. Hinnen, P. Wingfield, P. M. C. A. van Erd, A. de Reus and H. Schellekens, EMBO 3, 645-650 (1984).
DETD 256. Nagai, Y., T. Yoshida, M. Hamaguchi, H. Naruse, M. Iinuma, K. Maeno, and T. Matsumoto, Microbiol. Immunol. 24, 173-177 (1980).
DETD 261. Neurath, A. R., N. Strick and M. Girard, Mol. Immun. 26, 53-62 (1989).
DETD 262. Nixon, D. F., Townsend, A. R. M., Elvin, J. G., Rizza, C. R., Gallwey, J. and McMichael, A. J., Nature 326, 484-487 (1988).
DETD 263. Norrby, E., and Y. Gollmar, Infect. and Immun. 11, 231-239 (1975).
DETD 264. Norrby, E., and M. N. Oxman, Zn Fields Virology, 2nd Edition, eds. B. N. Fields and D. M. Knipe, (Raven Press, NY) pp. 1013-1044 (1990).
DETD 265. Norrby, E., S. N. Chen, T. Togashi, H. Shesberadaran, and K. P. Johnson, Archives of Virology 71, 1-11 (1982).
DETD 266. Nunberg, J. H., Williams, M. E., and Innis, M. A., J. Virol., 49, 629-632 (1984b).
DETD 267. Nunberg, J. H., Rodgers, J., GIlbert, J., and Snead, R. M., Proc. Natl. Acad. Sci. USA 81, 3675-3679 (1984a).
DETD 272. Oie, M., Shida, H., and Ichihashi, Y., Virology 176, 494-504 (1990).
DETD 277. Pachl, C., W. S. Probert, K. M. Hermsen, F. R. Masiarz, L. Rasmussen, T. C. Merigan, and R. R. Spaete, Virology 169, 418-426 (1989).
DETD 278. Paez, E., S. Dallo and M. Esteban, Proc. Natl. Acad. Sci. USA 82, 3365-3369 (1985).
DETD 279. Palumbo, G. J., D. J. Pickup, T. N. Fredrickson, L. J. McIntyre and

R. M. L. Buller, Virology 172, 262-273 (1989).
DETD 280. Palumbo, G. J., Pickup, D. J., Fredrickson, T. N., Mcintyre, L. J., and Buller, R. M. L., Virology 172, 262-273 (1989).
DETD 282. Panicali, D. and E. Paoletti, Proc. Natl. Acad. Sci. USA 79, 4927-4931 (1982).
DETD 284. Panicali, D., Davis, S. W., Mercer, S. R., and Paoletti, E., J. Virol. 37, 1000-1010 (1981).
DETD 285. Paoletti, E., B. Lipinskas, C. Samsonoff, S. Mercer and D. Panicali, Proc. Natl. Acad. Sci. USA 81, 193-197 (1984).
DETD 287. Parrish, C. R., Aguadro, C. F., and Carmichael, L. E., Virology 166, 293-307 (1988).
DETD 288. Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J-Y., and Mohammed, H. O., J. Virology 65, 6544-6552.
DETD 294. Perkus M. E., Piccini A., Lipinskas B. R., et al., Science 229, 981-984 (1985).
DETD 295. Perkus, M. E., Limbach, K., and Paoletti, E., J. Virol. 63, 3829-3836 (1989).
DETD 296. Perkus, M. E., D. Panicali, S. Mercer and E. Paoletti, Virology 152, 285-297 (1986).
DETD 297. Perkus, N. E., S. J. Goebel, S. W. Davis, G. P. Johnson, E. K. Norton and E. Paoletti, Virology 180, 406-410 (1991).
DETD 298. Perkus, N. E., Goebel, S. J., Davis, S. W., Johnson, G. P., Limbach, K., Norton, E. K., and Paoletti, E., Virology 179, 276-286 (1990).
DETD 299. Petrovskis, E. A., Timmins, J. G., Armentrout, M. A., Marchioli, C. C., Yancey, Jr., R. J., Post, L. E., J. Virol. 59, 216-223 (1986b).
DETD 300. Petrovskis, E. A., J. G. Timmins, and L. E. Post, J. Virol. 60, 185-193 (1986a).
DETD 301. Piccini, A., M. E. Perkus, and E. Paoletti, Methods in Enzymology 153, 545-563 (1987).
DETD 304. Plata, F., Autran, B., Martins, L. P., Wain-Hobson, S., Raphael, M., Mayaud, C., Denis, M., Guillon, J.-M., Debre, P., Nature 328, 348-351 (1987).
DETD 305. Plotkin, S. A., S. E. Starr, H. M. Friedman, E. Gonczol, and R. E. Weibel, J. Inf. Dis. 159, 860-865 (1989b).
DETD 306. Plotkin, S. A., H. M. Friedman, S. E. Starr, and E. Gonczol, In Contemporary Issues in Infectious Diseases, Vol. 8, eds. Root et al. (Churchill Livingstone, New York) pp. 65-92 (1989a).
DETD 307. Pontisso, P, M-A. Petit, M. J. Bankowski and M. E. Peeples, J. Virol. 63, 1981-1988 (1989).
DETD 308. Portetelle, D., Limbach,-K., Burny, A., Mammerickx, M., Desmetttre, P., Riviere, M., Zavada, J. and Paoletti, E. Vaccine 9, 194-200 (1991).
DETD 313. Rasmussen, L., M. Nelson, M. Neff, and T. C. Merigan, Jr., Virology 163, 308-318 (1988).
DETD 314. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R. Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Chrayeb, J., Chang, N. T., Gallo, R. C., and Wong-Staal, F., Nature 313,. . .
DETD 315. Rautmann, G., Kieny, M. P., Brandely, R., Dott, K., Girard, M., Montagnier, L., and Lecocq, J.-P., AIDS Research and Human Retroviruses 5, 147-157 (1989).
DETD 316. Rea, T. J., J. G. Timmins, G. W. Long, and L. E. Post, J. Virol. 54, 21-29 (1985).
DETD 319. Rice, C. M., Strauss, E. G., and Strauss, J. H., In The Togaviridae and **Flaviviridae**, eds. S. Schlesinger and M. J. Schlesinger, (Plenum Press, New York/London) pp. 279-326 (1986).
DETD 320. Rice, C. M., Lenches, E. M., Eddy, S. R., Shin, S. J., Sheets, R. L., and Strauss, J. H., Science 229, 726-733 (1985).
DETD 323. Rickinson, A. B., Rowe, M., Hart, I. J., Yao, Q. Y., Henderson, L. E., Rabin, H., and Epstein, N. A., Cell. Immunol. 87, 646-658 (1984).
DETD 324. Riviere Y., Tanneau-Salvadori, F., Regnault, A., Lopez, O., Sansonetti, P., Guy, B., Kieny, M.-P., Fournel, J. J. and Montagnier, L., J. Virol. 63, 2270-2277 (1989).

DETD 325. Robbins, A. K., Dorney, D. J., Wathen, M. W., Whealy, M. E., Gold, C., Watson, R. J., Holland, L. E., Weed, S. D., Levine, M., Gloricso, J. C., and Enquist, L. W., J. Virol. 61, 2691-2701 (1987).

DETD 326. Robbins, A. K., Watson, R. J., Whealy, M. A., Harp, W. W., and Enquist, L. W., J. Virol. 60, 436-449 (1986b).

DETD 328. Robbins, A. K., R. J. Watson, M. E. Whealy, W. W. Hays, and L. W. Enquist, J. Virol. 58, 339-347 (1986a).

DETD 331. Rojko, J. L., Hoover, E. A., Quackenbush, S. L., and Olsen, R. G., Nature 298, 385-388 (1982).

DETD 332. Romanos, M. A., Makoff, A. J., Fairweather, N. F., Beesley, K. M., Slater, D. E., Rayment, F. B., Payne, M. M., and Clare, J. J. Nucleic Acids Res. 19, 1461-1467 (1991).

DETD 333. Rooney F. F., Wohlenberg C., Cramer E. J. et al., J. Virol. 62, 1530-1534 (1988).

DETD 336. Ruegg, C. L., Monell, C. R., and Strand, M., J. Virol. 63, 3250--3256 (1989a).

DETD 338. Russell, M., S. Kidd, and M. R. Kelley, Gene 45, 333-338 (1986).

DETD 340. Saliki, J. T., Mizak, B., Flore, H. P., Gettig, R. R., Burand, J. P., Carmichael, L. E., Wood, H. A., and Parrish, C. R., J. Gen. Virol. (accepted) (1992).

DETD 341. Sambrook, J., Fritsch, E. F., and Maniatis, T., In Molecular Cloning: A Laboratory Manual, 2nd Edition, (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

DETD 342. Sanchez-Pescador, R., Power, M. D., Barr, P. J., Steimer, K. S., Stempien, M. M., Brown-Shimer, S. L., Gee, W., Renard, A., Randolph, A., Levy, J. A., Dina, D., and Luciw, P. A., Science 227,. . .

DETD 345. Sazawa H., Sugimori T., Morimoto T., Miura Y. and Watanabe M., Natl. Inst. Anim. Health Q. 9, 74-82 (1969).

DETD 349. Schlesinger, J. J., Brandriss, M. W., and Walsh, E. E., J. Immunol. 135, 2805-2809 (1985).

DETD 350. Schlesinger, J. J., Brandriss, M. W., Cropp, C. B., and Monath, T. P., J. Virol. 60, 1153-1155 (1986).

DETD 352. Schmaljohn, C. S., Jennings, G. B., Hay, J., Dalrymple, J. M., Virology 155, 633-643 (1986).

DETD 353. Schmaljohn, C. S., and Dalrymple, J. M., Virology 131, 482-491 (1983).

DETD 354. Schmaljohn, C. S., Schmaljohn, A. L., and Dalrymple, J. M., Virology 157, 31-39 (1987).

DETD 356. Schmaljohn, C. S., Chu, Y. K., Schmaljohn, A. L., and Dalrymple, J. M., J. Virology 64, 3162-3170 (1990).

DETD 357. Schmidt, D. M., Sidhu, N. K., Cianciolo, G. J., and Snyderman, R. (1987) Proc. Natl. Acad. Sci. USA 84, 7290-7294.

DETD . . . Sebring, R. W., Chu, H.-J., Chavez, L. G., Sandblom, D. S., Hustead, D. R., Dale, B., Wolf, D., Acree, W. M. (1991) JAVMA 199, 1413-1418.

DETD 360. Seligmann, E. B., In Laboratory Techniques in Rabies, (eds. M. M. Kaplan and H. Koprowski, (World Health Organization, Geneva) pp. 279-285 (1973).

DETD . . . S., AIDS Research and Human Retroviruses 5, 33-39 (1989). 362. Shapira, S. K., Chou, J., Richaud, F. V. and Casadaban, M. J., Gene 25, 71-82 (1983).

DETD 363. Shibley, G. P., Tanner, J. E., and Hanna, S. A., JAVMAk 199, 1402-1405 (1991).

DETD 364. Shida, H., Hinuma, Y., Hatanaka, M., Morita, M., Kidokoro, M., Suzuki, K., Maruyzam, T., Takahashi-Nishimaki, F., Sugimoto, M., Kitamura, R., Miyazawa, T., and Hayami, M., J. Virol. 62, 4474-4480 (1988).

DETD 366. Shida, H., T. Tochikura, T. Sato, T. Konno, K. Hirayoshi, M. Seki, Y. Ito, M. Hatanaka, Y. Hinuma, M. Sugimoto, F. Takahashi-Nishimaki, T. Naruyama, K. Miki, K. Suzuki, M. Morita, H. Sashiyama and M. Hayami, EMBO 6, 3379-3384 (1987).

DETD 369. Shope, R. E., In The Togaviruses, ed. R. W. Schlesinger, (Academic Press, New York) pp. 47-82 (1980).

DETD 370. Slabaugh, M. B. and N. A. Roseman, Proc. Natl. Acad. Sci. USA 86, 4152-4155 (1989).

DETD 371. Slabaugh, M., N. Roseman, R. Davis and C. Mathews, J. Virol. 62,

DETD 319 327 (1986).
DETD . . . and Yager P. A. A rapid tissue culture test for determining rabies neutralization antibody. In: Laboratory Techniques on Rabies. Eds. M. M. Kaplan and H. Koprowski.
DETD 373. Smith, G. L., M. Mackett and B. Moss, Nature 302, 490-495 (1983).
DETD 375. Smith, J. S., P. A. Yager and G. M. Baer, In Laboratory Techniques in Rabies, eds. M. M. Kaplan and H. Koprowski (WHO Geneva) pp. 354-357 (1973).
DETD 381. Stanberry, L. R., S. Kit and M. G. Myers, J. Virol. 55, 322-328 (1985).
DETD 384. Stewart, M. A., Warnock, M., Wheeler, A., Wiklie, N., Mullins, J. I., Oniono, D. E., and Neil, J. C. J. Virol. 58, 825-834 (1986).
DETD 387. Takahashi M., JK. Med. Entomol. 13, 275-284 (1976).
DETD 389. Tartaglia, J., Pincus, S., and Paoletti, E., Crit. Rev. Immunol. 10, 13-30 (1990).
DETD 390. Tartaglia, J., M. E. Perkus, J. Taylor, E. K. Norton, J. C. Audonnet, W. I. Cox, S. W. Davis, J. VanderHoeven, B. Meignier, M. Riviere, B. Languet, and E. Paoletti, Virology 188 (1992).
DETD 391. Tartaglia, J. and Paoletti, E., In Immunochemistry of viruses, II, eds. van Regenmortel, M. H. V. and Neurath, A. R., (Elsevier Science Publishers B.V., Amsterdam) pp. 125 (1990b).
DETD b 392. Taylor, J., Weinberg, R., Tartaglia, J., Richardson, C., Alkhatib, G., Briedis, D., Appel, M., Norton, E., and Poaletti, E., Virology 187, 321-328 (1992).
DETD 393. Taylor, J., Weinberg, R., Kawaoka, Y., Webster, R. G., and Paoletti, E., Vaccine 6, 504-508 (1988a).
DETD 394. Taylor, J., C. Trimarchi, R. Weinberg, B. Languet, F. Guillemin, P. Desmettre and E. Paoletti, Vaccine 9, 190-193 (1991b).
DETD 395. Taylor, J., S. Pincus, J. Tartaglia, C. Richardson, G. Alkhatib, D. Briedis, M. Appel, E. Norton and E. Paoletti, J. Virol. 65, 4263-4274 (1991c).
DETD 396. Taylor, J., Edbauer, C., Rey-Senelonge, A., Bouquet, J.-F., Norton, E., Goebel, S., Desmettre, P., and Paoletti, E. J. Virol. 64, 1441-1450 (1990).
DETD 397. Taylor, J., R. Weinberg, B. Languet, P. Desmettre, and E. Paoletti, Vaccine 6, 497-503 (1988).
DETD 398. Taylor, G., E. J. Stott, G. Wertz and A. Ball, J. Gen. Virol. 72, 125-130 (1991a).
DETD 402. Toyoda, T., T. Sakaguchi, K. Imai, N. M. Inocencio, B. Gotoh, M. Hamaguchi, and Y. Nagai, Virology 158, 242-247 (1987).
DETD 408. Valenzuela, P., D. Coit, M. A. Medina-Selby, C. H. Kuo, G. V. Nest, R. L. Burke, P. Bull, M. S. Urdea and P. V. Graves, Bio/Technology 3, 323-326 (1985).
DETD 409. Valenzuela, P., P. Gray, M. Quiroga, J. Zaldivar, H. M. Goodman and W. J. Rutter, Nature 280, 815-819 (1979).
DETD 410. Varma M. G., Pudney M., and Leeke C. J., Trans. R. Soc. Trop. Med. Hyg., 68, 374-382 (1974).
DETD 411. Vialard, J., M. Lalumiere, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson, J. Virol. 64, 37-50 (1990).
DETD 413. Wachsman , M., Aurelian, L., Smith, C., Lipinskas, B., Perkus, M. and Paoletti, E., J. Infect. Dis. 155, 1188-1197 (1987).
DETD 414. Waddell, G. H., Teigland, M. B., and Sigel, N. M., JAVMA 143, 587-590 (1963).
DETD 416. Walker, B. D., Flexner, C., Paradis, T. J., Fuller, T. C., Hirsch, M. S., Schooley, R. T. and Moss, B., Science 240, 64-66 (1988).
DETD . . . D., Chakrabarti, S., Moss, B., Paradi, T. J., Flynn, T., Durno, A. G., Blumberg, R. S., Kaplan, J. C., Hirsch, M. S., and Schooley, R. T., Nature 328, 345-348 (1987).
DETD 418. Wathen, M. W. and Wathen, L. M. K., J. Virol. 51, 57-62 (1984).
DETD 420. Watson, C. J., and Jackson, J. F., In: DNA Cloning, Vol. I., ed., Glover, D. M., (IRL Press, Washington, D.C.), pp. 79-88 (1985).
DETD 421. Waxham, M. N., Aronowski, J., Server, A. C., Walinsky, J. S., Smith, J. A., and Goodman, H. M., Virology 164, 318-325 (1988).
DETD 422. Waxham, M. N., Server, A. C., Goodman, H. M., and Walinsky, J. S., Virology 159, 381-388 (1987).
DETD 423. Weibel, R. E., In: Vaccines, eds. Plotkin, S. A., and Mortimer,

E. A., (W. B. Saunders)), pp. 225-254 (1988).

DETD 424. Weir, J., Bennett, **M.**, Allen, **E.**, Elkins, K., Martin, S. and Rouse, B., J. Gen. Virol. 70, 2587-2594 (1989).

DETD 432. Wiktor, T. J., S. A. Plotkin and H. Koprowski, In Vaccines, eds. Plotkin, S. A. and **E.** A. Mortimer (W. B. Saunders, Philadelphia), 474-491 (1988).

DETD 433. Wiktor, T. J., **E.** Gyorgy, H. D. Schlumberger, F. Sokol and H. Koprowski, J. Immunol. 110, 269-276 (1973).

DETD 434. Wiktor, T. J., R. I. Macfarlan, K. J. Reagan, B. Dietzschold, P. J. Curtis, W. H. Wunner, **M.** P. Kieny, R. Lathe, J. P. Lecocq, **M.** Mackett, B. Moss and H. Koprowski, Proc. Natl. Acad. Sci. USA 81, 7194-7198 (1984).

DETD 437. Wild, **T.** F., **E.** Malvoisin, and R. Buckland, J. Gen. Virol. 72, 439-447 (1991).

DETD 438. Winkler, G., Randolph, V. B., Cleaves, G. R., Ryan, T. **E.**, and Stollar, V., Virol 162, 187-196 (1988).

DETD 440. Wolff, L. H., Mathes, L. **E.**, and Osone, R. G., J. Immunol. Meth. 26, 151-156 (1979).

DETD 441. Wolinsky, J. S., and Waxhan, **M.** N., In: Virology, eds. Fields, B. N., and Knipe, D. **M.**, (Raven Press), pp. 989-1011 (1990).

DETD 443. -Wunsch, **M.**, Schultz, A. S., Koch, W., Friedrich, R., and Hunsmann, G., EMBO J. 2, 2239-2246 (1983).

DETD 445. Yamanishi, K., Dantas, J. R, Jr., Takahashi, **M.**, Yamanouchi, T., Damae, K., Takahoashi, Y., Tanishita, O., J. Virology 52, 231-237 (1984).

DETD 446. Yasuda, A., Kimura-Kuroda, J., Ogimoto, **M.**, Miyamoto, **M.**, Sata, T., Sato, T., Takamura, C., Kurata, T., Kojima, A., and Yasui, K., J. Virol. 64, 2788-2795 (1990).

DETD 447. Yelverton, **E.**, S. Norton, J. F. Obijeski and D. V. Goeddel, Science 219, 614-620 (1983)

DETD 448. Yoshida I., Takagi **M.**, Inokuma **E.**, Goda H., Ono K., Takaku K., and Oku J., Biken J. 24, 47-67 (1981).

DETD 452. Zanetti, A. R., **E.** Tanzi, L. Romano, P. Vigano, A. Cargnel, S. Hojvat and A. J. Zuckerman, J. Med. Virol. 32, 219-224 (1990).

DETD 453. Zarling, J. **M.**, Morton, W., Moran, P. A., McClure, J., Kosowski, S. G. and Hu, S.-L., Nature 323, 344-346 (1986).

DETD 455. Zhou, J., L. Crawford, L. McLean, X. Sun, **M.** Stanley, N. Almond and G. L. Smith, J. Gen. Virol. 71, 2185-2190 (1990).

DETD 456. Zingernagel, R. **M.**, Sato, T., Althage, A., and Kamisaku, H., Eur. J. Immunol. 14, 14-23 (1984).

DETD 457. Zweig, **M.**, Showalter, S., Bladen, S., Heilman, C. and Hampar, B., J. Virol. 47, 185-192 (1983).

. . . in claim 5 wherein the exogenous DNA is selected from the group consisting of rabies virus, Hepatitis B virus, Japanese **encephalitis virus**, **yellow fever virus**, **Dengue** virus, measles virus, pseudorabies virus, Epstein-Barr virus, herpes simplex virus, human immunodeficiency virus, simian immunodeficiency virus, equine herpes virus, bovine. . .

L14 ANSWER 10 OF 15 USPATFULL on STN

1998:64734 Modified recombinant vaccinia virus and expression vectors thereof.

Paoletti, Enzo, Delmar, NY, United States
Perkus, Marion E., Altamont, NY, United States
Taylor, Jill, Albany, NY, United States
Tartaglia, James, Schenectady, NY, United States
Norton, Elizabeth K., Latham, NY, United States
Riviere, Michel, Ecully, France
de Taisne, Charles, Lyon, France
Limbach, Keith J., Troy, NY, United States
Johnson, Gerard P., Waterford, NY, United States
Pincus, Steven E., East Greenbush, NY, United States
Cox, William I., Troy, NY, United States
Audonnet, Jean-Christophe Francis, Albany, NY, United States
Gettig, Russell Robert, Averill Park, NY, United States
Virogenetics Corporation, Troy, NY, United States (U.S. corporation)
US 5762938 19980609

APPLICATION: US 1996-709209 19960821 (8)
DOCUMENT TYPE: Utility; Granted.
CAS INDEXING IS AVAILABLE FOR THIS PATENT.

AB What is described is a modified vector, such as a recombinant poxvirus, particularly recombinant vaccinia virus, having enhanced safety. The modified recombinant virus has nonessential virus-encoded genetic functions inactivated therein so that virus has attenuated virulence. In one embodiment, the genetic functions are inactivated by deleting an open reading frame encoding a virulence factor. In another embodiment, the genetic functions are inactivated by insertional inactivation of an open reading frame encoding a virulence factor. What is also described is a vaccine containing the modified recombinant virus having nonessential virus-encoded genetic functions inactivated therein so that the vaccine has an increased level of safety compared to known recombinant virus vaccines.

CLM What is claimed is:

1. A recombinant vaccinia virus wherein regions C7L-K3L, J2R, B13R+B14R, A26L, A56R and I4L have been deleted therefrom.

2. A recombinant vaccinia virus wherein regions C23L-F4L, J2R, B13R+B14R, A26L, A56R and I4L have been deleted therefrom.

3. A recombinant vaccinia virus wherein regions C7L-K1L, J2R, B13R-B29R, A26L, A56R and I4L have been deleted therefrom.

4. A recombinant vaccinia wherein regions C23L-F4L, J2R, B13R-B29R, A26L, A56 and I4L have been deleted therefrom.

5. The recombinant vaccinia virus of claim 1 including exogenous DNA from a non-vaccinia source.

6. The recombinant vaccinia virus of claim 2 including exogenous DNA from a non-vaccinia source.

7. The recombinant vaccinia virus of claim 3 including exogenous DNA from a non-vaccinia source.

8. The recombinant vaccinia virus of claim 4 including exogenous DNA from a non-vaccinia source.

9. A recombinant vaccinia virus selected from the group consisting of: vP954, vP938, vP953, vP977, vP996, vP1006 and vP1015.

10. A method for expressing a gene product in a cell cultured in vitro, which method comprises introducing into the cell the recombinant vaccinia virus as claimed in any of claims 1, 2, 3, 4, 5, 6, 7, 8 or 9, and culturing the cell under appropriate conditions for expression of the gene product.

11. An immunological composition comprising a carrier and a recombinant vaccinia virus as claimed in any of claims, 1, 2, 3, 4, 5, 6, 7, 8, 8 or 9.

AI US 1996-709209 19960821 (8) <--

SUMM . . . sequence to be inserted into the virus, particularly an open reading frame from a non-pox source, is placed into an **E.** coli plasmid construct into which DNA homologous to a section of DNA of the poxvirus has been inserted. Separately, the. . . flanking a region of pox DNA containing a nonessential locus. The resulting plasmid construct is then amplified by growth within **E.** coli bacteria (Clewell, 1972) and isolated (Clewell et al., 1969; Maniatis et al., 1982).

SUMM Second, the isolated plasmid containing the DNA gene sequence to be inserted is transfected into a cell culture, **e.**g. chick embryo fibroblasts, along with the poxvirus. Recombination between homologous pox DNA in the plasmid and the viral genome respectively. . .

SUMM Cowpox virus (Brighton red strain) produces red (hemorrhagic) pocks on

the chorioallantoic membrane of chicken eggs. Spontaneous deletions within the cowpox genome generate mutants which produce white pocks (Pickup et al., 1984). The. . .

DRWD FIG. 26 shows the nucleotide sequence of FeLV-B **Envelope** Gene (SEQ ID NO:310);

DETD . . . from Bethesda Research Laboratories, Gaithersburg, Md. New England Biolabs, Beverly, Mass.; and Boehringer Mannheim Biochemicals, Indianapolis, Ind. Klenow fragment of **E**. coli polymerase was obtained from Boehringer Mannheim Biochemicals. BAL-31 exonuclease and phage T4 DNA ligase were obtained from New England. . .

DETD . . . NcoI site (pos. 172,253) were removed by digestion of pSD419 with NcoI/SmaI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation generating plasmid pSD476. A vaccinia right flanking arm was obtained by digestion of pSD422 with HpaI. . . ID NO:6/SEQ ID NO:7) ##STR3## generating pSD479. pSD479 contains an initiation codon (underlined) followed by a BamHI site. To place **E**. coli Beta-galactosidase in the B13-B14 (u) deletion locus under the control of the u promoter, a 3.2 kb BamHI fragment. . .

DETD . . . at the pUC/vaccinia junction was destroyed by digestion of pSD478 with EcoRI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation, generating plasmid pSD478E$^-$. pSD478E$^-$ was digested with BamHI and HpaI and ligated with annealed synthetic oligonucleotides. . .

DETD . . . XbaI within vaccinia sequences (pos. 137,079) and with HindIII at the pUC/vaccinia junction, then blunt ended with Klenow fragment of **E**. coli polymerase and ligated, resulting in plasmid pSD483. To remove unwanted vaccinia DNA sequences to the right of the A26L. . . digestion with BglII (pos. 140,136) and with SARI at the pUC/vaccinia junction, followed by blunt ending with Kienow fragment of **E**. coli polymerase and ligation. The resulting plasmid was designated pSD489. The 1.8 kb ClaI (pos. 137,198)/EcoRV (pos. 139,048) fragment from. . .

DETD A 3.3 kb BglII cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD A 3.2 kb BglII/BamHI (partial) cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD . . . were removed from the pUC/vaccinia junction by digestion of pSD466 with EcoRI/BamHI followed by blunt ending with Klenow fragment of **E**. coli polymerase and ligation. Recombination between vP708 and pSD467 resulted in recombinant vaccinia deletion mutant, vP723, which was isolated as. . .

DETD . . . SphI and religated, forming pSD451. In pSD451, DNA sequences to the left of the SphI site (pos. 27,416) in HindIII **M** are removed (Perkus et al., 1990). pSD409 is HindIII **M** cloned into pUC8.

DETD To provide a substrate for the deletion of the [C7L-K1L] gene cluster from vaccinia, **E**. coli Beta-galactosidase was first inserted into the vaccinia M2L deletion locus (Guo et al., 1990) as follows. To eliminate the. . . unique BglII site inserted into the M2L deletion locus as indicated above. A 3.2 kb BamHI (partial)/BglII cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the 11 kDa promoter (Bertholet et al., 1985) was. . .

DETD . . . deleted for vaccinia genes [C7L-K1L] was assembled in pUC8 cut with SmaI, HindIII and blunt ended with Klenow fragment of **E**. coli polymerase. The left flanking arm consisting of vaccinia HindIII C sequences was obtained by digestion of pSD420 with XbaI (pos. 18,628) followed by blunt ending with Klenow fragment of **E**. coli polymerase and digestion with BglII (pos. 19,706). The right flanking arm consisting of vaccinia HindIII K sequences was obtained. . .

DETD . . . coding sequences, pSD518 was digested with BamHI (pos. 65,381) and HpaI (pos. 67,001) and blunt ended using Klenow fragment of **E**. coli polymerase. This 4.8 kb vector fragment was ligated with a 3.2 kb SmaI cassette containing the **E**. coli Beta-galactosidase gene (Shapira et al., 1983) under the control of the vaccinia 11 kDa promoter (Bertholet et al., 1985;. . .

DETD . . . al., 1989) was inserted into pCE13 by digesting pCE13 with

SalI, filling in the sticky ends with Klenow fragment of **E**. coli DNA polymerase and digesting with HindIII. A HindIII-EcoRV fragment containing the H6 promoter sequence was then inserted into pCE13. . .

DETD . . . H6 promoted NDV-F cassette by cloning a HindIII fragment from pCE59 that had been filled in with Klenow fragment of **E**. coli DNA polymerase into the HpaI site of pCE71 to form pCE80. Plasmid pCE80 was completely digested with NdeI and. . .

DETD In NDV-infected cells, the F glycoprotein is anchored in the **membrane** via a hydrophobic transmembrane region near the carboxyl terminus and requires post-translational cleavage of a precursor, F_0, into two disulfide. . .

DETD . . . that immunoreactive proteins were presented on the infected cell surface. To determine that both proteins were presented on the plasma **membrane**, mono-specific rabbit sera were produced against vaccinia recombinants expressing either the F or HN glycoproteins. Indirect immunofluorescence using these sera. . .

DETD . . . mutagenized expression cassette contained within pRW837 was derived by digestion with HindIII and EcoRI, blunt-ended using the Klenow fragment of **E**. coli DNA polymerase in the presence of 2 mM dNTPs, and inserted into the SmaI site of pSD513 to yield. . .

DETD . . . into pRW843 (containing the measles HA gene). Plasmid pRW843 was first digested with NotI and blunt-ended with Klenow fragment of **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The resulting plasmid, pRW857, therefore contains the measles virus F. . .

DETD Immunoprecipitation reactions were performed as previously described (Taylor et al., 1990) using a guinea-pig anti measles serum (Whittaker **M**. A. Bioproducts, Walkersville, Md.).

DETD . . . The site-directed mutagenesis was done using MRSYN5 (SEQ ID NO:52) (5'-GCGAGCGAGGCCATGCATCGTGCGAATGGCCCC-3') and MRSYN6 (SEQ ID NO:53) (5'-GGGGGGACGCGCGGGTCTAGAAGGCCCCGCCTGGCGG-3') and selection on **E**. coli dut^{31} ung^{31} strain. CJ236 (International Biotechnologies, Inc., New Haven, Conn.). Mutagenesis was performed according to the protocols of Kunkel. . .

DETD . . . A 1.4 kb fragment containing the I3L promoter/PRV gp50 gene was isolated and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs.

DETD Vero cells were infected at an **m**.o.i. of 10 pfu per cell with the individual recombinant viruses, with the NYVAC parent virus, or were mock infected. After. . . were dissociated with RIPA buffer (1% NP-40, 1% Na-deoxycholate, 0.1% SDS, 0.01M methionine, 5 mM EDTA, 5 mM 2-mercapto-ethanol, 1 **m**/ml BSA, and 100 u/ml aprotinin). Samples analyzed with sheep anti-gpIII and a monoclonal specific for gp50 were lysed in 1×. . .

DETD Extraneous 3'-noncoding sequence was then eliminated from pGC10. This was accomplished by recircularizing the **E**. coli DNA polymerase I (Klenow fragment) filled-in 4,900 bp SalI-SmaI (partial) fragment of pGC10. The plasmid generated by this manipulation. . .

DETD Extraneous DNA was then eliminated. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 6,000 bp HindIII-BamHI (partial) fragment of pGBCD1, containing the H6-promoted gB, gC and. . .

DETD Vero cells were infected at an **m**.o.i. of 10 pfu per cell with recombinant vaccinia virus, with the NYVAC parent virus (vP866) or were mock infected. After. . .

DETD . . . inserted individually into three different sites of the virus. The three HBV genes encode the following protein products: (1) HBV **M** protein, (referred to here as small pre S antigen, or spsAg), (2) HBV L protein (referred to here as large. . .

DETD The synthetic S1+S2 region was assembled in five double stranded sections A through **E** as indicated above using synthetic oligonucleotides, MPSYN290 through MPSYN308 (SEQ ID NO:90)-(SEQ ID NO:99), as set out below. Oligonucleotides ranged. . . within a section were kinased before annealing of the section. Sequence of synthetic oligonucleotides used to construct sections A through **E** are given below. Only the coding strand is shown. Relevant restriction sites are noted. Initiation codons for S1 (section A), S2 (section C) and core

(Section **E**) are underlined. Section A, MPSYN290 294 (SEQ ID NO:90)-(SEQ ID NO:92) ##STR26## The vaccinia I3L promoter was synthesized using pMP1,a subclone. . .

DETD Construction of pRW838 is illustrated below. Oligonucleotides A through **E**, which overlap the translation initiation codon of the H6 promoter with the ATG of rabies G, were cloned into pUC9 as pRW737. Oligonucleotides A through **E** contain the H6 promoter, starting at NruI, through the HindIII site of rabies G followed by BglII. Sequences of oligonucleotides A through **E** (SEQ ID NO:109)-(SEQ ID NO. 113) are: ##STR30## The diagram of annealed oligonucleotides A through **E** is as follows: ##STR31##

DETD oligonucleotides A through **E** were kinased, annealed (95° C. for 5 minutes, then cooled to room temperature), and inserted between the PvuII sites of. . .

DETD . . . stages of assembly of mature rabies virus particles, the glycoprotein component is transported from the golgi apparatus to the plasma **membrane** where it accumulates with the carboxy terminus extending into the cytoplasm and the bulk of the protein on the external surface of the cell **membrane**. In order to confirm that the rabies glycoprotein expressed in ALVAC-RG was correctly presented, immunofluorescence was performed on primary CEF. . .

DETD The initial inoculation was performed at an **m**. o. i. of 0.1 pfu per cell using three 60mm dishes of each cell line containing 2×10^6 cells per dish.. . .

DETD . . . parental canarypox virus, (b) ALVAC-RG, the recombinant expressing the rabies G glycoprotein or (c) vCP37, a canarypox recombinant expressing the **envelope** glycoprotein of feline leukemia virus. Inoculations were performed under ketamine anaesthesia. Each animal received at the same time: (1) 20. . .

DETD (**e**) Primary CEF cells.

DETD . . . electrophoresis the viral DNA band was visualized by staining with ethidium bromide. The DNA was then transferred to a nitrocellulose **membrane** and probed with a radiolabelled probe prepared from purified ALVAC genomic DNA.

DETD . . . seed 23 3.34

Vaccine Batch H
23 4.52

Vaccine Batch I
23 3.33

Vaccine Batch K
15 3.64

Vaccine Batch L
15 4.03

Vaccine Batch **M**
15 3.32

Vaccine Batch N
15 3.39

Vaccine Batch J
23 3.42

---

[a] Expressed as mouse LD_{50}

[b] Expressed as $\log_{10}$ $TCID_{50}$

DETD . . . 1.7 2.2 2.2 2.2

39	vCP37[d]	NT	<1.2	<1.2	1.7	2.1	2.2	N.T.[g]
55	vCP37[d]	NT	<1.2	<1.2	1.7	2.2	2.1	N.T.
37	ALVAC-RG[e]	2.2	<1.2	<1.2	3.2	3.5	3.5	3.2
53	ALAC-RG[e]	2.2	<1.2	<1.2	3.6	3.6	3.6	3.4
38	ALVAC-RG[f]	2.7	<1.7	<1.7	3.2	3.8	3.6	N.T.
54	ALVAC-RG[f]	3.2	<1.7	<1.5	3.6.	.	.	28 after primary vaccination

[c] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of ALVAC
[d] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of vCP37
[e] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of ALVACRG
[f] Animals received 5.0 $\log_{10}$ $TCID_{50}$ of ALVACRG
[g] Not tested.

DETD TABLE 15

Inoculation of chimpanzees with ALVAC-RG

Weeks post-Inoculation	Animal 431 I.M.	Animal 457 S.C.
0	<8[a]	<8
1	<8	<8
2	8	32
4	16	32
8	16	32
12[b] /0	16	8
13/1	128	128
15/3	256	512
20/8	64. . .	

DETD Construction of NYVAC Recombinants Expressing **Flavivirus** Proteins

DETD This example describes the construction of NYVAC donor plasmids containing genes from Japanese **encephalitis virus** (JEV), **yellow fever virus** (YF) and **Dengue** type 1,the isolation of the corresponding NYVAC **Flavivirus** recombinants and the ability of vaccinia recombinants expressing portions of the genomes of JEV or YF to protect mice against. . .

DETD . . . and AccI fragment of JEV2 (Mason et al., 1991) containing the plasmid origin and JEV cDNA encoding the carboxy-terminal 40% **prM** and amino-terminal two thirds of **E** (nucleotides 602 to 2124), generating plasmid JEV20 containing JE sequences from the ATG of C through the SacI site (nucleotide 2124) found in the last third of **E**.

DETD . . . 1991) in which TTTTTGT nucleotides 1304 to 1310 were changed to TCTTGT), containing JE sequences from the last third of **E** through the first two amino acids of NS2B (nucleotides 2124 to 4126), the plasmid origin and vaccinia sequences, was ligated. . .

DETD . . . end] generated plasmid JEV25 which contains JE cDNA extending from the SacI site (nucleotide 2124) in the last third of **E** through the carboxy-terminus of **E**. The SacI-EagI fragment from JEV25 was ligated to the SacI-EagI fragment of JEV8 (containing JE cDNA encoding 15 aa C, **prM** and amino-terminal two thirds of **E** nucleotides 337 to 2124, the plasmid origin and vaccinia sequences) yielding plasmid JEV26. A unique SmaI site preceding the ATG. . .

DETD . . . fragment from JEV7 (Mason et al., 1991) yielded JEV29 (containing a SmaI site followed by JE cDNA encoding 30 aa **E**, NS1, NS2A nucleotides 2293 to 4126) and JEV30 (containing a SmaI site followed by JE cDNA encoding 30 aa **E**, NS1, NS2A, NS2B nucleotides 2293 to 4512).

DETD HeLa cell monolayers were prepared in 35 mm diameter dishes and infected with vaccinia viruses (**m**.o.i. of 2 pfu per cell) or JEV (**m**.o.i. of 5 pfu per cell) before radiolabeling. Cells were pulse labeled with medium containing ^{35} S-Met and chased for 6. . .

DETD Recombinant vP825 encoded the capsid protein, structural protein precursor **prM**, the structural glycoprotein **E**, the nonstructural glycoprotein NS1, and the nonstructural protein NS2A (McAda et al., 1987). Recombinant vP829 encoded the putative 15 aa **signal sequence** preceding the amino-terminus of **prM**, as well as **prM**, and **E** (McAda et al., 1987). Recombinant vP857 contained a cDNA encoding the 30 aa hydrophobic carboxy-terminus of **E**, followed by NS1 and NS2A. Recombinant vP864 contained a cDNA encoding the same proteins as vP857 with the addition of NS2B. In recombinants vP825 and vP829 a potential vaccinia virus early transcription termination signal in **E** (TTTTTGT;

nucleotides 1504-1510) was modified to TCTTGT without altering the aa sequence. This change was made in an attempt to increase the level of expression of **E** since this sequence has been shown to increase transcription termination in in vitro transcription assays (Yuen et al., 1987).

DETD **E** and **prM** Are Correctly Processed When Expressed By Recombinant Vaccinia Viruses.

DETD Pulse-chase experiments demonstrate that proteins identical in size to **E** were synthesized in cells infected with all recombinant vaccinia viruses containing the **E** gene (Table 16). In the case of cells infected with JEV, vP555 and vP829, an **E** protein that migrated slower in SDS-PAGE was also detected in the culture fluid harvested from the infected cells (Table 16). This extracellular form of **E** produced by JEV- and vP555-infected cells contained mature N-linked g:Lycans (Mason, 1989; Mason et al., 1991), as confirmed for the extracellular forms of **E** produced by vP829-infected cells. Interestingly, vP825, which contained the C coding region in addition to **prM** and **E** specified the synthesis of **E** in a form that is not released into the extracellular fluid (Table 16).

DETD Immunoprecipitations prepared from radiolabeled recombinant vaccinia-infected cells using a MAb specific for **M** (and **prM**) revealed that **prM** was synthesized in cells infected with vP555, vP825, and vP829, and **M** was detected in the culture fluid of cells infected with vP555 or vP829 (Table 16).

DETD . . . (data not shown). This result indicated that vP829 infected cells produce extracellular particles similar to the empty viral envelopes containing **E** and **M** observed in the culture fluids harvested from vP555 infected cells (Table 16 and Mason et al., 1991).

DETD Pre-challenge sera pooled from selected animals in each group were tested for their ability to immunoprecipitate radiolabeled **E** and NS1. The results of these studies (Table 16) demonstrated that: (1) the magnitude of immune response induced to **E** was vP829>vP555>vP825, (2) all viruses encoding NS1 and NS2A induced antibodies to NS1, and (3) all immune responses were increased. . . sera collected from these animals (Table 17) confirmed the results of the immunoprecipitation analyses, showing that the immune response to **E** as demonstrated by RIP correlated well with these other serological tests (Table 17).

DETD TABLE 16

Characterization of proteins expressed by vaccinia recombinants expressing JEV proteins and their immune responses

	vP555	vP829	vP825	vP857	vP864
Proteins expressed[a]					
Intracellular	**prM, E** NS1	**prM, E** NS1	**prM, E** NS1	NS1	NS1
secreted	**M, E**, NS1	**M, E**	none	NS1	NS1
Particle formation[b]	+	+	-	-	-
Immune response[c]					
single	**E**	**E**	NS1	NS1	NS1
double	**E**, NS1	**E**	**E**, NS1	NS1	NS1

[a] Radiolabelled cell lysates and culture fluids from vaccinia virus JEV recombinant infected ells were harvested and JEVspecific proteins immunoprecipitated using mAbs to **E**, **M** and NS1 proteins.

[b] Formation of extracellular particles with HA activity as described in the text.

[c] JEV proteins were. . .

DETD . . . isolated and ligated to a SacI (JEV nucleotide 2124) to EagI fragment of JEV25 (containing the remaining two thirds of **E**,

translation stop and TSN1, generating 6EV56. 6EV56 was transfected into vP866 (NYVAC) infected cells to generate the vaccinia recombinant vP923. . .

DETD Plasmid YFO containing YF cDNA encoding the carboxy-terminal 80% **prM, E** and amino-terminal 80% NS1 (nucleotides 537-3266) was derived by cloning an AvaI to NsiI fragment of YF cDNA (nucleotides 537-1659). . . and KpnI digested IBI25 (International Biotechnologies, Inc., New Haven, Conn.). Plasmid YF1 containing YF cDNA encoding C and amino-terminal 20% **prM** (nucleotides 119-536) was derived by cloning a RsaI to AvaI fragment of YF cDNA (nucleotides 166-536) and annealed oligonucleotides SP46. . . and YF nucleotides 119-165) into AvaI and HindIII digested IBI25. Plasmid YF3 containing YF cDNA encoding the carboxy-terminal 60% of **E** and amino-terminal 25% of NS1 was generated by cloning an ApaI to BamHI fragment of YF cDNA (nucleotides 1604-2725) into. . . cDNA (nucleotides 4339-4940) into SacI and XbaI digested IBI25. Plasmid YF13 containing YF cDNA encoding the carboxy-terminal 25% of C, **prM** and amino-terminal 40% of **E** was derived by cloning a BAlI to ApaI fragment of YF cDNA (nucleotides 384-1603) into ApaI and SmaI digested IBI25.

DETD . . . in YF1 (TTTTTCT nucleotides 263-269 and TTTTTGT nucleotides 269-275) to (SEQ ID NO:122) TTCTTCTTCTTGT creating plasmid YF1B, (2) in the **E** gene in YF3 (nucleotides 1886-1893 TTTTTTGT to TTCTTTGT 189 aa from the carboxy-terminus and nucleotides 2429-2435 TTTTTGT to TTCTTGT 8. . . YF3C (nucleotides 1965-2725) was exchanged for the corresponding fragment of YF3B generating YF4 containing YF cDNA encoding the carboxy-terminal 60% **E** and amino-terminal 25% NS1 (nucleotides 1604-2725) with both mutagenized transcription.termination signals. An ApaI to BamHI fragment from YF4 (nucleotides 1604-2725) was substituted for the equivalent region in YFO creating plasmid YF6 containing YF cDNA encoding the carboxy-terminal 80% **prM, E** and amino-terminal 80% NS1 (nucleotides 537-3266) with both mutagenized transcription termination signals. Plasmid YF6 was digested with EcoRV within the. . .

DETD . . . described above was used (1) to insert XhoI and ClaI sites preceding the ATG 17 aa from the carboxy-terminus of **E** (nucleotides 2402-2404) in plasmid YF3C creating YF5, (2) to insert XLoI and ClI sites preceding the ATG 19 aa from the carboxy-terminus of **prM** (nucleotides 917-919) in plasmid YF13 creating YF14, (3) to insert an XhoI site preceding the ATG 23 aa from the carboxy-terminus of **E** (nucleotides 2384-2386) in plasmid YF3C creating plasmid YF25, (4) and to insert an XhoI site and ATG (nucleotide 419) in. . .

DETD . . . YF5 (nucleotides 1604-2725) was exchanged for the corresponding region of YFO creating YF7 containing YF cDNA encoding the carboxy-terminal 80% **prM, E** and amino-terminal 80% NS1 (nucleotides 537-3266) with XhoI and ClaI sites at 2402 (17 aa from the carboxy-terminus of **E**) and a mutagenized transcription termination signal at 2429-2435 (8 aa from the carboxy-terminus of **E**). The kpaI to **E**,HI fragment from YF25 (nucleotides 1604-2725) was exchanged for the corresponding region of YFO generating YF26 containing YF cDNA encoding the carboxy-terminal 80% **prM, E** and amino-terminal 80% NS1 (nucleotides 537-3266) with an XhoI site at nucleotide 2384 (23 aa from the carboxy-terminus of **E**) and mutagenized transcription termination signal at 2428-2435 (8 aa from the carboxy-terminus of **E**).

DETD . . . YF14 (nucleotides 537-1603) was substituted for the corresponding region in YF6 generating YF15 containing YF cDNA encoding the carboxy-terminal 80% **prM, E** and amino-terminal 80% NS1 (nucleotides 537-3266) with XhoI and ClaI sites at nucleotide 917 (19 aa from the carboxy-terminus of **prM**) and two mutagenized transcription termination signals. YF6 was digested within IBI25 with EcoRV and within YF at nucleotide 537 with. . .

DETD . . . from YF12 encoding carboxy-terminal 20% NS1, NS2A and NS2B (nucleotides 3267-4569), XhoI to KpnI fragment from YF15 encoding 19 aa **prM, E** and amino-terminal 80% NS1 (nucleotides 917-3266) and XhoI-SmaI digested pHES4 were ligated generating YF23. An XhoI to BamHI fragment from YF26 encoding 23 aa **E**, amino-terminal 25% NS1 (nucleotides 2384-2725) was liga-ted to an XhoI to BamHI fragment from

YF25 (containing the carboxy terminal 75% NS1). . .

DETD XhoI-SmaI digested pHES4 was ligated to a purified XhoI to KpnI fragment from YF7 encoding 17 aa **E** and amino-terminal 80% NS1 (nucleotides 2402-3266) plus a KpnI to SmaI fragment from YF10 encoding the carboxy-terminal 20% NS1 and NS2A (nucleotides 3267-4180) creating YF18. An XhoI to BamHI fragment from YF2 encoding C, **prM**, **E** and amino-terminal 25% NS1 (nucleotides 119-2725) was ligated to a XhoI to hamHI fragment of YF18 (containing the carboxy-terminal 75%. . . the origin of replication and vaccinia sequences) generating YF20. A XhoI to BamHI fragment from YF46 encoding 21 aa C, **prM**, **E** and amino-terminal 25% NS1 (nucleotides 419-2725) was ligated to the XhoI to BamHI fragment from YF18 generating YF47. Oligonucleotide SP46. . .

DETD Recombinant vP725 encoded the putative 17-aa **signal sequence** preceding the N terminus of the nonstructural protein NS1 and the nonstructural proteins NS1 and NS2A (Rice et al., 1985). Recombinant vP729 encoded the putative 19-aa **signal sequence** preceding the N terminus of **E**, **E**, NS1, NS2A and NS2B (Rice et al., 1985). Recombinant vP764 encoded C, **prM**, **E**, NS1, NS2A and NS2B (Rice et al., 1985). Recombinant vP766 encoded C, **prM**, **E**, NS1 and NS2A (Rice et al., 1985). Recombinant vP869 encoded the putative 21-aa **signal sequence** preceding the N terminus of the **prM** structural protein precursor as well as **prM**, **E**, NS1 and NS2A (Rice et al., 1985).

DETD A XhoI to SmaI fragment from YF47 (nucleotides 419-4180) containing YF cDNA encoding 21 amino acids C, **prM**, **E**, NS1, NS2A (with nucleotide 2962 missing in NS1) was ligated to XhoI-SmaI digested SPHA-H6 (HA region donor plasmid) generating YF48.. . . (nucleotide 3262) and a 6700 bp fragment isolated (containing the plasmid origin of replication, vaccinia sequences, 21 amino acids C, **prM**, **E**, amino-terminal 3.5% NS1, carboxy-terminal 23% NS1, NS2A) and ligated to a SacI-Asp718 fragment from YF18 (containing the remainder of NS1. . . in YF51 were removed using oligonucleotide-directed double-strand break mutagenesis (Mandecki, 1986) creating plasmid YF50 encoding YF 21 amino acids C, **prM**, **E**, NS1, NS2A in the HA locus donor plasmid. Donor plasmid YF50 was transfected into vP866 (NYVAC) infected cells to generate. . .

DETD . . . double-strand break mutagenesis creating YF49. Oligonucleotide-directed mutagenesis (Kunkel, 1985) was used to insert a SmaI site at the carboxy-terminus of **E** (nucleotide 2452) in YF4 creating YF16. An ApaI-SmaI fragment of YF49 (containing the plasmid origin of replication, vaccinia sequences and YF cDNA encoding 21 amino acid C, **prM**, and amino-terminal 43% **E**) was ligated to an ApaI-SmaI fragment from YF16 (nucleotides 1604-2452 containing the carboxy-terminal 57% **E**) generating YF53 containing 21 amino acids of C, **prM**, **E** in the HA locus. Donor plasmid YF53 was transfected into vP913 (NYVAC-MV) infected cells to generate the vaccinia recombinant vP997.

DETD . . . digested pUC8. An EcoRI-HindIII fragment from DEN1 (nucleotides 2559-3745) and SacI-EcoRI fragment of DEN cDNA encoding the carboxy-terminal 36% of **E** and amino-terminal 16% NS1 (nucleotides 1447-2559, Mason et al., 1987b) were ligated to HindIII -SacI digested IBI24 (International Biotechnologies, Inc., New Haven, Conn.) generating DEN3 encoding the carboxy-terminal 64% **E** through amino-terminal 45% NS2A with a base missing in NS1 (nucleotide 2467).

DETD . . . an AvaI-SacI fragment of DEN cDNA (nucleotides 424-1447 Mason et al., 1987) generating DEN4 encoding the carboxy-terminal 11 aa C, **prM** and amino-terminal 36% **E**.

DETD Plasmid DEN6 containing DEN cDNA encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 (nucleotides 1447-2579 with nucleotide 2467 present Mason et al., 1987b) was derived by cloning a SacI-XhoI. . . Biotechnologies, Inc., New Haven, Conn.). Plasmid DEN15 containing DEN cDNA encoding 51 bases of the DEN 5' untranslated region, C, **prM** and amino-terminal 36% **E** was derived by cloning a HindIII-SacI fragment of DEN cDNA (nucleotides 20-1447, Mason et al., 1987b) into HindIII-SacI digested IBI25.. . .

DETD . . . change the following potential vaccinia virus early transcription termination signals (Yuen et al., 1987). The two T5NT seqeunces in the **prM** gene in DEN4 were mutagenized (1) 29 aa from the

carboxy terminus (nucleotides 822-826 HTTTCT to TATTCT) and (2) 15 aa. . .

DETD . . . 4102) in plasmid DEN23 creating DEN24, (2) to insert a SmaI site and ATG 15 aa from the carboxy-terminus of **E** in DEN7 (nucleotide 2348) creating DEN10, (3) to insert an EagI and HindIII site at the carboxy-terminus of NS2B (nucleotide. . .

DETD . . . DEN7 (nucleotides 1447-2579) was substituted for the corresponding region in DEN3 generating DEN19 containing DEN cDNA encoding the carboxy-terminal 64% **E** and amino-terminal 45% NS2A (nucleotides 1447-3745) with nucleotide 2467 present and the modified transcription termination signal (nucleotides 2448-2454). A XhoI-XbaI. . .

DETD A HindIII-PstI fragment of DEN16 (nucleotides 20-494) was ligated to a HindIII-PstI fragment from DEN47 (encoding the carboxy-terminal 83% **prM** and amino-terminal 36% of **E** nucleotides 494-1447 and plasmid origin of replication) generating DEN17 encoding C, **prM** and amino-terminal 36% **E** with part of the H6 promoter and EcoRV site preceding the amino-terminus of C. A HindIII-BglII fragment from DEN17 encoding the carboxy-terminal 13 aa C, **prM** and amino-terminal 36% **E** (nucleotides 370-1447) was ligated to annealed oligonucleotides SP111 and SP112 (containing a disabled IiindIII sticky end, EcoRV site to -1. . . a BglII sticky end) creating DEN33 encoding the EcoRV site to -1 of the H6 promoter, carboxy-terminal 20 aa C, **prM** and amino-terminal 36% **E**.

DETD . . . digested pTP15 (Mason et al., 1991) was ligated to a SmaI-SacI fragment from DEN4 encoding the carboxy-terminal 11 aa C, **prM** and amino-terminal 36% **E** (nucleotides 377-1447) and SacI-,EagI fragment from DEN3 encoding the carboxy-terminal 64% **E**, NS1 and amino-terminal 45% NS2A generating DENL. The SacI-XhoI fragment from DEN7 encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 (nucleotides 1447-2579) was :Ligated to a BstEII-SacI fragment from DEN47 (encoding the carboxy-terminal 55% **prM** and amino-terminal 36% **E** nucleotides 631-1447) and a BstEII-XhoI fragment from DENL (containing the carboxy-terminal 11 aa C, amino-terminal 45% **prM**, carboxy-terminal 82% NS1, carboxy-terminal 45% NS2A, origin of replication and vaccinia sequences) generating DEN8. A unique SmaI site (located between. . .

DETD . . . an EcoRV-SacI fragment of DEN8VC (containing vaccinia sequences, H6 promoter from -21 to -124, origin of replication and amino-terminal 64% **E**, NS1, amino-terminal 45% NS2A nucleotides 1447-3745) generating DEN18. A XhoI-EagI fragment from DEN25 (nucleotides 2579-4102) was ligated to an XhoI-EagI fragment of DEN18 (containing the origin of replication, vaccinia sequences and DEN C, **prM**, **E** and amino-terminal 18% NS1 nucleotides 68-2579) generating DEN26. An EcoRV-SacI fragment from DEN8VC (positions -21 to -1 H6 promoter DEN. . . to an EcoRV-SacI fragment of DEN26 (containing the origin of replication, vaccinia sequences and DEN region encoding the carboxy-terminal 64% **E**, NS1 and NS2A with a base missing in NS1 at nucleotide 2894) generating DEN32. DEN32 was transfected into vP410 infected. . .

DETD . . . DEN10 (nucleotides 1447-2579) was substituted for the corresponding region in DEN3 generating DEN11 containing DEN cDNA encoding the carboxy-terminal 64% **E**, NS1 and amino-terminal 45% NS2A with a SmaI site and ATG 15 aa from the carboxy-terminus of **E**. A SmaI-EagI fragment from DEN11 (encoding the carboxy-terminal 15 aa **E**, NS1 and amino-terminal 45% NS2A nucleotides 2348-3745) was ligated to SmaI-EgaI digested pTP15 generating DEN12.

DETD . . . EcoRV-XhoI fragment from DEN8VC (positions -21 to -1 H6 promoter DEN nucleotides 377-2579 encoding the carboxy-terminal 11 aa C, prN **E**, amino-terminal 18% NS1) was ligated to an EcoRV-XhoI fragment from D:EN31 (containing the origin of replication, vaccinia sequences and DEN. . . An EcoRV-SacI fragment from DEN33 (positions -21 to -1 H6 promoter DEN nucleotides 350-1447 encoding the carboxy-terminal 20 aa C, **prM** and amino-terminal 36% **E**) and a SacI-XhoI fragment from DEN32 (encoding the carboxy-terminal 64% **E** and amino-terminal 18% NS1 nucleotides 1447-2579) were ligated to the EcoRV-SacI fragment from DEN31 described above generating DEN34. DEN34 was. . .

DETD . . . the left terminus of vaccinia and by introducing a deletion near the right terminus. All deletions were accomplished using the **E**. coli guanine phosphoribosyl transferase gene and mycophenolic acid in a transient selection system.

DETD For use as a selectable marker, the **E**. coli gene encoding guanine phosphoribosyl transferase (Ecogpt) (Pratt et al., 1983) was placed under the control of a poxvirus promoter.. . .

DETD . . . subunit of ribonucleotide reductase (Slabaugh et al., 1988). Also included in this deletion is ORF F2L, which shows homology to **E**. coli dUTPase, another enzyme involved in nucleotide metabolism (Goebel et al., 1990a,b). F2L also shows homology to retroviral protease (Slabaugh. . .

DETD . . . seguences, the predicted translation product of Copenhagen ORF B16 is truncated at the amino terminus and does not contain a **signal sequence**. B19R encodes a vaccinia surface protein (S antigen) expressed at early times post infection (Ueda et al., 1990). Both B16R. . .

DETD . . . immunological assays was comprised of RPMI 1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 20 mM HEPES (N-2--hydroxyethylpiperazine-N'-2-ethanesulfonate), 5×10^{-5} **M** 2-mercaptoethanol, 100 IU penicillin per ml, and 100 µg/ml streptomycin. Stim Medium was comprised of Eagle's Minimum Essential Medium supplemented with 10% FBS, 4 mM L-glutamine, 10^{-4} **M** 2-mercaptoethanol, 100 IU penicillin per ml, and 100 µg streptomycin per ml.

DETD ALVAC and NYVAC Recombinants Containing the V3 Loop and Epitope 88 of the HIV-1 (IIIB) **Envelope**.

DETD . . . isolated by phenol extraction (2×) and ether extraction (1×). The isolated fragment was blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The fragment was ligated to pSD550, a derivative of pSD548. . .

DETD ALVAC- and NYVAC-Based Recombinants Expressing the HIV-1 (IIIB) **Envelope** Glycoproteins.

DETD . . . pBSHIV3BEAII was digested with NruI and XbaI. The derived 2.7 kb fragment was blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This fragment contains the entire HIV-1 env gene juxtaposed 3'. . .

DETD . . . followed by a partial KpnI digestion. The 1.6 kb fragment was blunt-ended by treatment with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2mM dNTPs. This fragment was inserted into pSD54IVC digested with SmaI to yield. . .

DETD . . . Vero cells monolayers were either mock, infected, infected with the parental virus vP866, or infected with recombinant virus at an **m**.o.i. of 10 PFU/cell. Following a 1 hr adsorption period, the inoculum was aspirated and the cells were overlayed with 2. . .

DETD . . . using sera pooled from HIV-1 seropositive individuals showed specific precipitation of the gp120 and gp41 mature forms of the gp160 **envelope** glycoprotein from vP911 infected cell lysates. No such specific gene products were detected in the parentally (NYVAC; vP866) infected cell. . .

DETD . . . for 1 hour in tissue culture medium containing 2% FBS at 37° C. with the appropriate vaccinia virus at a **m**.o.i. of 25 pfu per cell. Following infection, the stimulator cells were washed several times in Stim Medium and diluted to. . .

DETD . . . cells were infected overnight by incubation at 1×10^{7} cells per ml in tissue culture medium containing 2% FBS at a **m**.o.i. of 25 pfu per cell for 1 hour at 37° C. Following incubation, the cells were diluted to between $1\text{-}2\times10^{6}$. . .

DETD . . . * -4.0

	±1.8	2.2	1.2
vP911	-4.0	4.6 *	1.4
	±2.5	2.0	5.1
vP921	-3.4	10.7 *	15.5 *
	±0.9	1.5	2.8

E:T = 100:1

$P < 0.05$ vs appropriate controls, Student's t-test

DETD . . . plasmid vector, pIBI25 (International Biotechnologies, Inc., New Haven, Conn.), generating plasmid pIBI25env. Recombinant plasmid pIBI25env was used to transform competent **E**. coli CJ236 (dut- ung-) cells. Single-stranded DNA was isolated from phage derived by infection of the transformed **E**. coli CJ236 cells with the helper phage, MG408. This single-stranded template was used in vitro mutagenesis reactions (Kunkel et al.,. . .

DETD . . . 2.5 kb (envIS+) and 2.4 kb (envIS-), respectively, were isolated and blunt-ended by reaction with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mMdNTPs. These fragments were ligated with the 3.5 kb fragment derived by digestion of pSIVenvVV with NruI and pstI with a subsequent blunting step with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The plasmid pSIVenvVV contains the SIV env gene expression cassette. . .

DETD . . . seropositive individuals were performed as described in Materials and Methods. All six recombinants directed the synthesis of the HIV-1 gp161 **envelope** precursor. The efficiency of processing of gp160 to gp120 and gp41, however, varied between cell types and was also affected. . .

DETD . . . to yield pBSH6HIV2ENV. The 2.7 kb HindIII/XbaI insert from pBSH6HIV2ENV was isolated and blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2mM DNTP. The blunt-ended fragment was inserted into a SmaI digested pSD5HIVC insertion. . .

DETD . . . gp160. Vero cell monolayers were either mock infected, infected with the parental virus vP866, or infected with vP920 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD Human sera from HIV-2 seropositive individuals specifically precipitated the HIV-2 gp160 **envelope** glycoprotein from vP920 infected cells. Furthermore, the authenticity of the expressed HIV-2 env gene product was confirmed, since the gp160. . .

DETD . . . coding sequence juxtaposed 3' to the vaccinia virus H6 promoter. This fragment was blunted with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The blunt-ended fragment was ligated to SmaI digested PSDSHIVC to. . .

DETD . . . digestion with HindIII liberated a 2.7 kb HindIII/EcoRI fragment. This fragment was blunt-ended by treatment with Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. The fragment was ligated into pSD550VC digested with SmaI. The. . .

DETD The SIV gp140 env gene product is a typical glycoprotein associated with the plasma **membrane** of infected cells. It is expressed as a polyprotein of 140 kDa that is proteolytically cleaved to an extracellular species. . .

DETD . . . and gag) in Vero cells infected with the NYVAC/HIV recombinants was analyzed by immunoprecipitation. Vero cells were infected at an **m**.o.i. of 10 with the individual recombinant viruses, with the NYVAC parent virus, or were mock infected. After a 1 hour. . .

DETD The plasmid pF7D3 was linearized with XhoI and blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This linearized plasmid was ligated with annealed oligonucleotides F7MCSB (SEQ. . .

DETD . . . the H6 promoter) and PstI. The 3.5 kb resultant fragment was isolated and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This blunt-ended fragment was ligated to a 1700 bp EcoRV/HpaI. . .

DETD . . . HA molecule is synthesized and glycosylated as a precursor molecule at the rough endoplasmic reticulum. During passage to the plasma **membrane** it undergoes extensive post-translational modification culminating in proteolytic cleavage into the disulphide linked HA_1, and HA_2 subunits and insertion into the host cell **membrane** where it is subsequently incorporated into mature viral envelopes. To determine whether the HA molecules produced in cells infected with. . .

DETD . . . end EcoRV fragment (D). Plasmid pVHAH6g3 was digested with BglII and KpnI to isolate the 1330 bp BglII-H6-EHV-1 gC 5'-KpnI fragment (**E**).

DETD Fragments C, D and **E** were finally ligated together into vector pSD541VC digested with BglII and XhoI to produce plasmid pJCA042. Plasmid pJCA042 is the. . .

DETD . . . fragment (L). Plasmid pVHAH6g13 was digested with BglII and XhoI to isolate the 440 bp BglII-H6-EHV-1 gC 5' portion-XhoI fragment (**M**). Fragments K, L and **M** were then ligated together to produce plasmid pJCA040.

DETD . . . authentic BHV1 gIV glycoprotein. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1051 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gene was then cloned into pIBR8. This was accomplished by cloning the 2,285 bp StuI fragment of pIBRS6 into the **E**. coli DNA polymerase I (Klenow fragment) filled-in 4,300 bp StuI-BglII (partial) fragment of pIBR8. The plasmid generated by this manipulation. . .

DETD The H6-promoted BHV1 gI gene was then moved to a vaccinia virus donor plasmid. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 2,900 bp BglII-NcoI (partial) fragment of pIBR20 into the SmaI site of pSD542.. . .

DETD . . . gI and gIV glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1074 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . authentic BHV1 gIII glycoprotein. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1073 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gIII and gIV glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1083 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gI and gIII glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1087 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . gIII and gIV glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP1079 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1 (obtained from Eurogentec, Liege, Belgium; Renard et al., European Patent Application No:86870095) with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . gE1 and gE2 glycoproteins. Vero cell monolayers were either mock infected, infected with NYVAC or infected with vP972 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD . . . cloned into pIB125. This was accomplished by blunt-ending the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1, containing the gE1 "gene", with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . cloned into pIBI25. This was accomplished by blunt-ending the 1,370 bp EcoRI-BamHI fragment of pSP65-gE1, containing the gE1 "gene", with **E**. coli DNA polymerase I (Klenow fragment), ligating XhoI linkers onto the ends and cloning the resulting fragment into the XhoI. . .

DETD . . . pig polyclonal serum followed by fluorescein isothiocyanate goat anti-guinea pig. Cells infected with vP1001 showed gB expressed on the plasma **membrane**. Weak internal expression was detected within cells infected with vCP139.

DETD . . . gene was excised from pED3 with NruI and XhoI and the purified fragment was cloned into pVQH6CP3L (plasmid described in **Flavivirus** section) cut with NruI and XhoI. The resulting plasmid, pC3-VP2,

contains the H6 promoted VP2 gene flanked by the C5. . .

DETD . . . with EcoRI, which recognizes a unique EcoRI site within the canarypox sequences, and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase. The resultant plasmid was designated as pCPCV1. This plasmid contains the vaccinia virus H6 promoter followed by. . .

DETD An M13 clone containing the hemagglutinin (HA) gene from equine influenza virus (A2/Suffolk/89) was provided by Dr. **M**. Binns (Animal Health Trust, P.O. Box 5, Newmarket, Suffolk, CB8 7DW, United Kingdom). This clone contains a full-length 1.7 kb. . .

DETD . . . recombination tests with vP425 as the rescuing virus to construct a recombinant vaccinia virus (vP453) which expresses the entire FeLV **envelope** glycoprotein.

DETD . . . tests with vP410 as the rescuing virus to generate vP456. This vaccinia virus recombinant was generated to express the entire **envelope** glycoprotein lacking the putative immunosuppressive region.

DETD . . . of the H6 promote sequence. The PstI site is located 420 bp downstream from the translation termination signal for the **envelope** glycoprotein open reading frame.

DETD . . . of the H6 promoter sequence. The HpaI site is located 180 bp downstream from the translation termination signal for the **envelope** glycoprotein open reading frame. These isolated fragments were blunt-ended. These 2.2 kbp H6/FeLV env sequences were inserted into the nonessential. . .

DETD . . . with EcoRI, which recognizes a unique EcoRI site within the canarypox sequences, and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase. The resultant plasmid was designated as pCPCV1. This plasmid contains the vaccinia virus H6 promoter followed by. . .

DETD The putative immunosuppressive region is situated within the p15E transmembrane region of the FeLV **envelope** glycoprotein (Cianciolo et al., 1986; Mathes et al., 1978). This region was deleted in the following manner. The FeLV-A env. . .

DETD . . . into the SmaI site of pSD553. This insertion was performed following blunt-ending the fragment with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs.

DETD Vero cell monolayers were infected at an **m**.o.i. equal to 10 pfu/cell with parental or recombinant viruses. At 1 hr post-infection, the inoculum was aspirated and methionine-free medium. . .

DETD In order to determine whether the env gene product expressed by vCP83 and vCP87 was transported to the plasma **membrane** of infected cells, immunofluorescence experiments were performed as described previously (Taylor et al., 1990). Primary CEF monolayers were infected with. . .

DETD . . . to challenge with reline leukemia virus

		Time (weeks) relative to challenge						
	Cat	-5	-2	0	+3	+6	+9	+12
Group	No.	**E**[1] V[2]						
			EV	EV	EV	F[3] EV	FEV	FEV
1. vCP 93: Felv-A	1	--	--	--	++	-++	+++	+++
	2. . .	++	-++	+++	+++			
	17	--	--	--	++	-++	+++	+++
	18	--	--	--	++	-++	+++	+++

*__E__ = FeLV p27 antigen in plasma (ELISA)
V = infectious virus in plasma (virus isolation)
F = FeLV antigen in. . .

DETD The FHV-1 CO strain genomic DNA was completely digested with EcoRI and the fragment **M** (4470 bp) was excised from the agarose gel (Geneclean procedure) and cloned into vector pBS-SK+ digested with EcoRI and phosphatased. The resulting plasmid containing the FHV-1 EcoRI **M** fragment was designated pHFeM2. The FHV-1 EcoRI **M** fragment complete nucleotide sequence for both strands was obtained from several subclones of the FHV-1 EcoRI **M** fragment inserted into vector pBS-SK+, using the modified T7 enzyme Sequenase (U.S. Biochemical Corp.) (Tabor and

Richardson, 1987). Standard dideoxynucleotide. . .

DETD . . . the FHV-1 gD 5'-most region were confirmed by direct sequencing of pJCA071. Plasmid pJCA067 is a subclone of FHV-1 EcoRI **M** fragment. It has been generated as follows. Plasmid pHFeM2 was digested with BamHI and the 1850 bp BamHI-BamHI fragment was. . .

DETD Expression of the Hantaan virus G1 and G2 glycoproteins was accomplished by insertion of the **M** segment into the NYVAC and ALVAC vectors under the control of the entomopoxvirus 42 kDa promoter. The poxvirus expression cassette. . .

DETD A cDNA clone of the Hantaan virus **M** segment was derived as described by Schmaljohn et al. (1987) and provided by Dr. J. Dalrymple (Virology Division, U.S. Army. . . full sequence of the cDNA was presented previously by Schmaljohn et al. (1987). The 326 bp 5'-most region of the **M** segment coding sequence was derived using the plasmid pTZ19R containing the **M** segment cDNA as template and oligonucleotides HM5P (SEQ ID NO:335) (5'-ATGGGGA TATGGAAGTGG-3') and HM3P (SEQ ID NO:336) (5'-CATGTT CCTTTCAAGTCAAC-3'). This. . .

DETD The 3'-most 748 bp of the **M** segment coding sequence was derived by PCR using the cDNA clone contained in pTZ19R as template and oligonucleotides HMTS-5 (SEQ. . .

DETD The plasmid containing the **M**-specific cDNA clone in pTZ19R was used to transform GM48 (Dam^-) bacterial cells (BRL, Gaithersburg, Md.). Plasmid DNA derived from this. . . the 42 kDa promoter fused to the 5' most region of the coding sequence. The resultant plasmid containing the entire **M** segment expression cassette was designated as pBSHVM. The entire **M** segment cassette was excised from PBSHVM using restriction endonucleases HindIII and EcoRI. The 3508 bp derived fragment was blunt-end using the Klenow fragment of the **E**. coli in the presence of 2 mM dNTPs. The blunt ended fragment was inserted into pSD550 to yield pHVMVC.

DETD . . . vP882. Recombinant virus was identified by in situ hybridization according to standard procedures (Piccini et al., 1987) using a radiolabeled **M**-specific DNA probe. Recombinant plaques were purified by 3 rounds of plaque purification and amplified for further analysis. Recombinant virus, vP882, contains the Hantaan **M** segment in the I4L locus of vaccinia virus. Replacement of the I4L open reading frame with the **M** segment cassette in the vP804 background creates a NYVAC- equivalent virus background (Tartaglia et al., 1992).

DETD The 3508 bp HindIII/EcoRI fragment derived from pBSHVM, containing the **M** segment cassette (above), was inserted into pC4I digested with HindIII and EcoRI. The plasmid pC4I was derived as follows. A. . .

DETD Insertion of the **M** segment cassette into pC4I yielded plasmid pC4HVM. The plasmid pC4HVM was linearized with SmaI for insertion of a 100 bp. . . pC4HVMVQ was digested with SmaI followed by a subsequent partial HindIII digestion to recover a 3.6 kb fragment containing the **M** segment cassette. This fragment was blunt-ended using the klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This blunt-ended fragment was inserted into SmaI digested pSPCPC3L to. . .

DETD . . . identify and to purify the recombinant virus (as above; Piccini et al., 1987). The ALVAC-based recombinant containing the Hantaan virus **M** segment was designated as vCP114.

DETD . . . by linearization with XbaI followed by a partial HindIII digestion. This fragment was blunt-ended using the klenow fragment of the **E**. coil DNA polymerase in the presence of 2 mM dNTPs and then inserted into the SmaI site of pSD541 (defined. . .

DETD . . . in the ATI site and vP951 contains this cassette at the same locus, but by virtue of rescue with the **M** segment containing vP882, also contains the **M** segment in the I4L locus.

DETD The plasmid pBSHVM was linearized with SalI and blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs. This was ligated to the 1.4 kb XbaI/partial HindIII (Blunt-ended. . . from pBSHVS containing the Hantaan S segment expression cassette. The derived plasmid was designated as pBSHVMS. This plasmid contained the **M** and S cassettes in a head to head configuration. Plasmid pBSHVMS was linearized with XhoI, blunted with Klenow (as above),. . .

DETD . . . a 1.9 kb fragment containing the S segment expression cassette. This fragment was blunt-ended using the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs and inserted into the SmaI site of pSPCP3L (defined in. . .

DETD Expression Analysis of the NYVAC-AND ALVAC- Based Hantaan Virus **M** and S Segment Recombinants

DETD . . . Schmaljohn (Virology Division, U.S. Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, Md.). The recombinant viruses containing the **M** segment alone (vP882, and vCP114) or in combination with the S segment (vP951) displayed intense surface fluorescence using any of. . .

DETD . . . inoculated with NYVAC-based Hepatitis B virus (HBV) recombinants vP856, vP930, vP932 and vP975 (Example 13). vP856 expresses spsAg, the middle (**M**) form of the surface antigen. vP930 expresses lpsAg, the large (L) form of the surface antigen. vP932 expresses both spsAg. . .

DETD TABLE 34

AUSAB and CORAB
Rabbits
Analysis of sera of rabbits inoculated with NYVAC-based HBV recombinants expressing
the middle (**M**) form of the surface antigen (small pre S antigen), the large (L) form
of the surface antigen (large pre S antigen) and. . . 2 regions fused to the core antigen.

#	vP	HBV genes	week 1	2	3	4	5	6	7	8
AUBAB[a]										
A133	932	**M** + L	>512	>512	262	352	13000	6500	3600	5400
A134	932	**M** + L	250	235	72	80	3900	561	800	218
A135	975	**M** + L + S/C	36	58	274	406	1300	646	436	268
A136	975	**M** + L + S/C	103	>512	127	136	13468	4698	3168	2768
CORAB[b]										

A135 975
M + L + S/C
80 20 20 80 320 80 320
80
A136 975
M + L + S/C
20 5 5 5 80 80 320
80

Rabbits were inoculated with 10^8 pfu of the. . .
DETD TABLE 35

Pre-S2 ELISA
Rabbits
Analysis by ELISA of sera from rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form of the surface antigen, the large (L) form of the surface antigen and a fusion protein (S/C) consisting of the pre. . . 2 regions fused to the core antigen.

#	vP	HBV genes	week 1	2	3	4	5	6	7	8
A133										
932		M + L	0	0	29	35	474			
							602	358		
									419	
A134										
932		M + L	0	0	0	277	2017			
							3099			
								847		
									500	
A135										
975		M + L + S/C								
			0	0	0	0	175			
							105	94		
									48	
A136										
975		M + L + S/C								
			0	0	0	0	2440			
							763	672		
									355	

Rabbits were inoculated by the intramuscular (IM) route. . .
DETD TABLE 36

Pre-S1 ELISA
Rabbits
Analysis by ELISA test of sera from rabbits inoculated with NYVAC-based HBV recombinants expressing the middle (M) form and the large (L) form of the surface antigen and the preS1 + 2/core fusion protein (S/C).

#	vP	HBV genes	week 0	2	4	5	6	8
A133	932	M + L	<10	<10	<10	<10	<10	<10
A134	932	M + L	<10	<10	<10	<10	17	<10
A135	975	M + L + S/C	<10	<10	15	40	<10	24
A136	975	M + L + S/C	15	16	17	117	52	49

Rabbits were inoculated by the intradermal (ID) route with 10^8. . .
DETD TABLE 37

Pre-S2 ELISA
Guinea Pigs

Analysis by ELISA of sera from guinea pigs inoculated with
NYVAC-based HBV recombinants expressing the middle (**M**) form
of the surface antigen, the large (L) form
of the surface antigen and the preS1 + 2/core fusion protein (S/C).

#	vP	HBV genes	week 0	5	6
85	856	**M**	<10	<10	<10
86	856	**M**	<10	<10	<10
87	930	L	<10	46	35
88	930	L	<10	30	93
89	932	**M** + L	<10	39	<10
90	932	**M** + L	<10	33	19
91	975	**M** + L + S/C	<10	22	84
92	975	**M** + L + S/C	<10	53	269

Guinea pigs were inoculated by the SC route with 10^8 of the indicate
. . .

DETD TABLE 38

CORAB
Mice
Analysis of sera by CORAB test of mice inoculated with vaccinia
recombinant vP975 expressing the HBV middle (**M**) form of the surface
antigen, the large (L) form of the surface antigen and a fusion protein
(S/C) consisting of the pre. . . 2 regions fused to the core antigen.

Group	vP	HBV genes	Week 1	2	3	4	5	6	7	8
D	975	**M** + L + S/C	-[a]	-	-	-	5	5	5	5

Mice were inoculated by the IM route with. . .

DETD TABLE 39

Pre-S2 ELISA
Mice
Analysis by ELISA of sera from mice inoculated with
NYVAC-based HBV recombinants expressing the middle (**M**) form
of the surface antigen, the large (L) form of the surface antigen
and the preS1 + 2/core fusion protein (S/C).

	vP	HBV genes	week 0	5	6
Group A	856	**M**	<10	73	70
Group B	930	L	<10	93	112
Group C	932	**M** + L	<10	970	1146
Group D	975	**M** + L + S/C	<10	1054	1062

Groups of eight or twelve mice were inoculated by the IM route with. . .

DETD TABLE 40

Analysis by ELISA of sera from mice inoculated with
NYVAC-based HBV recombinants expressing the middle (**M**) form
of the surface antigen, the large (L) form of the surface antigen
and the preS1 + 2/core fusion protein (S/C)

	vP	HBV genes	week 0	5
Group B	903	L	60	244
Group C	932	**M** + L	66	125
Group D	975	**M** + L + S/C	63	1554

Groups of eight or twelve mice were inoculated by the IM route with 10^7. . .

DETD . . . monolayers were either infected with parental virus, CPpp (ALVAC) or vP866 (NYVAC), or infected with vCP1661 or vP1075 at an **m**.o.i. of 10 pfu/cell. Cells were infected, incubated in modified Eagle's medium (minus methionine) containing [^{35} S]-methionine (20 µCi/ml), lysed and. . .

DETD . . . by Makoff et al., 1989) for fragment C produced by papain digestion of native tetanus toxin as well as an **E**. coli produced recombinant fragment C which is identical to that encoded by vCP161 and vP1075.

DETD . . . days post-challenge. NYVAC-based pseudorabies virus recombinant viruses were all shown to reduce the effects of the virulent pseudorabies virus challenge (i.**e**. clinical signs and virus isolation) compared to the controls, with the gp50 expressing recombinant virus being the most efficacious. In. . .

DETD . . . receiving vP1015

A168	<1.3[d]						
		<1.3	<1.3	1.3[c]			
					2.2	2.2	2.2
A169	<1.3	1.6	1.6	1.6	3.1	3.1	2.5
Animals receiving vP913							
A116	<1.3	<1.3	N.D[e]				
				<1.3	2.8	2.2	2.2
A117	<1.3	<1.3	N.D.	<1.3	1.9	1.9	1.9

[a] Day of inoculation with 8.0 $\log_{10}$ pfu of. . . highest dilution showing a 50% reduction in plaque number as compared to preinoculation serum.

[d] Lowest dilution tested was 1:20

[e] Not done

DETD Construction of Insertion Vector Containing Japanese **Encephalitis Virus** (JEV) 15aaC, **prM**, **E**, NS21, NS2A

DETD . . . promoter, plasmid origin of replication and C5 flanking arms isolated. Plasmid JEVL14VC containing JEV cDNA encoding 15 amino acids C, **prM**, **E**, NS1, NS2A in a vaccinia virus donor plasmid (Mason et al., 1991) (nucleotides 337-4125, Konishi et al., 1991) was digested. .

.

DETD Construction of C5 Insertion Vector Containing JEV 15aaC, **prM**. **E**

DETD . . . annealed oligonucleotides SP131 (SEQ ID NO:382) and SP132 (SEQ ID NO:383) (containing a SphI sticky end, T nucleotide completing the **E** coding region, translation stop, a vaccinia early transcription termination signal (AT5AT; Yuen and Moss, 1987), a second translation stop, and XbaI sticky end) generating plasmid JEVCP5 which encodes 15 amino acids C, **prM** and **E** under the control of the H6 promoter between C5 flanking arms.

DETD JEVCP1 was transfected into ALVAC infected primary CEF cells to generate the canarypox recombinant vCP107 encoding 15 amino acids C, **prM**, **E**, NS1, NS2A. JEVCP5 was transfected into ALVAC infected primary CEF cells to generate the canarypox recombinant vCP140 encoding JEV 15 aa C, **prM** and **E**. ##STR49##

DETD Immunoprecipitation experiments were performed as described previously (Konishi et al., 1991). The **E** protein produced in vCP107 and vCP140 infected cells comigrates with the **E** protein produced by JEV-vaccinia recombinants which have been shown to produce an authentic **E** protein (Konishi et al., 1991). vCP107 produces an NS1 protein that comigrates with the NS1 protein produced by JEV-vaccinia recombinants. . .

DETD TABLE 48

Protective efficacy of TROVAC-NDV (vFP96) in SPF and commercial broiler chickens.

Bird Group	NDV HI GMT[d]	Percent Protection[e]

Dose	Week 3	Week 4 NDV		
Group 1[a]				
2.0	<5	<5	70	100
4.0	<5	<5	70	100

None. . . with prior history of vaccination with fowlpox virus

[c] Specific pathogen free birds

[d] Geometric mean titer of HI antibody

[e] Percent protection of birds after NDV or Fowpox challenge

DETD . . . centrifugation and resuspended in Assay Medium (RPMI 1640 containing 10% fetal bovine serum, 20 mM HEPES, 2 mM L-glutamine, 5×10^{-5} **M** 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin). For memory CTL activity, the spleen cells from immunized mice were resuspended in Stimulation Medium (Minimum Essential Medium with Earle's salts containing 10% fetal bovine serum, 2 mM L-glutamine, 10^{-4} **M** 2-mercaptoethanol, 100 U/ml penicillin, and 100 µg/ml streptomycin) and stimulated in vitro in upright 25 cm^2 tissue culture flasks with. . . titrated effector cells in 96-well microtiter plates for a 4 hr ^{51} Cr release assay. Effector to target cell ratios (**E**:T) shown for the three assays were 100:1 (primary), 20:1 (memory), and 50:1 (secondary). Percent cytotoxicity was calculated as (experimental ^{51}. . .

DETD . . . and resuspended in the original volume of Cytotoxicity Medium, divided into two equal portions with or without complement (Rabbit Lo-Tox **M**, Cedarlane) and incubated at 37° C. for 45 min. The cells were then washed in Assay Medium and, based on. . .

DETD . . . apparent molecular masses of 160 kDa, 120 kDa, and 41 kDa, respectively. These are consistent with expression of the precursor **envelope** glycoprotein (160 kDa) and the proteolytically derived mature forms (120 kDa and 41 kDa).

DETD . . . digested pC5L to yield pC5HIV3BEEC. A 2.7 kb NruI/XbaI fragment from pBSHIV3BEECM was blunt-ended with the Kienow fragment of the **E**. coli DNA polymerase and inserted into NruI/SmaI digested pSPHAH6 to yield pHAHIV3BEEC.

DETD . . . by isolating the 2.1 kb NruI/XbaI fragment from pBSHIIMT. This fragment was then blunt-ended with the Klenow fragment of the **E**. coli DNA polymerase in the presence of 2 mM dNTPs and inserted into pSPHAH6 digested with NruI and SmaI to. . .

DETD . . . Corp., Emeryville, Calif.). Investigation of surface immunofluorescence indicated that vCP138 and vP1035 infected cells contained HIV-1(MN) gp120 in the plasma **membrane**. significantly, the surface staining of VCP138 and vP1035 infected cells was greatly enhanced compared to cells infected with recombinant viruses (i.**e**. vCP125, vCP124, vP1004,and vP1008) expressing gp160 or a non-anchored gp120 . Results from immunoprecipitation analyses confirmed the expression of gp120. . .

DETD . . . precursor protein. Vero cell monolayers were either mock infected, infected with the parental virus or infected with vP969 at an **m**.o.i. of 10 PFU/cell. Following an hour adsorption period, the inoculum was aspirated and the cells were overlayed with 2 mls. . .

DETD An H6-promoted truncated HIV-1 **envelope** gene was then inserted into pHIVG4. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of pHIVE10, containing an H6-promoted truncated HIV-1 **envelope** gene, into the filled-in SUSHI site of pHIVG4. The plasmid generated by this manipulation is called pHIVGE11.

DETD . . . New Haven, Conn.). The plasmid pBSHIV3BCDT1 contains an H6 promoted cassette to express a severely truncated form of the HIV-1(IIIB) **envelope** (amino acid 1 to 447; Ratner et al., 1985). Expression of this cassette was evaluated to eliminate CD4 binding while. . .

DETD An H6-promoted truncated HIV-1 **envelope** gene was then inserted into pHIVG7. This was accomplished by cloning the **E**. coli DNA polymerase I (Klenow fragment) filled-in 1,600 bp XhoI-NotI fragment of PHIVEIO